A cixiid survey for natural potential vectors of '*Candidatus* Phytoplasma phoenicium' in Lebanon and preliminary transmission trials

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Running title: Potential cixiid vectors of '*Ca*. Phytoplasma phoenicium'

ABSTRACT

Almond witches'-broom (AlmWB) disease, associated with '*Candidatus* Phytoplasma phoenicium', is an emerging threat with real risk of introduction in Euro-Mediterranean Countries. Its rapid spread over large geographical areas suggests the presence of efficient insect vector(s). In the present work, a survey on cixiids was carried out in Lebanon in the years 2010-2013 in AlmWB-infested almond and nectarine orchards. Insects were collected by means of different methods, identified with a stereo microscope, and analyzed for phytoplasma identification through 16S rDNA PCR-based amplification and nucleotide sequence analyses. Preliminary transmission trials were performed with the most abundant species.

A list of the cixiid genera and species present in the studied area is given as well as some information about their biology. '*Ca.* Phytoplasma phoenicium' strains were detected in the genera *Cixius*, *Tachycixius*, *Eumecurus*, and *Hyalesthes*. Preliminary trials revealed that *Tachycixius* specimens were able to transmit the detected strains to healthy peach potted seedlings. Further studies are required to better clarify the taxonomic status and the bio-ethology of collected planthoppers and deeply study their role as phytoplasma vectors.

Keywords: almond witches'-broom; planthoppers; *Prunus* sp.; weeds; 16S rDNA

INTRODUCTION

Fruit tree diseases, caused by phytoplasmas, represent an increasing threat in Europe and in the Mediterranean Basin (Janse, 2012). During the last two decades, the outbreak of a lethal devastating almond (*Prunus amygdalus* Batsch) disease, named almond witches'-broom (AlmWB), has led to a rapid decline of almond trees in Northern Lebanon (Choueiri *et al.,* 2001, Abou-Jawdah *et al.,* 2002,) and Iran (Salehi *et al.,* 2006). AlmWB was also detected in peach (*P. persica*) and nectarine (*P. persica* var. *nucipersica*) in southern Lebanon (Abou-Jawdah *et al.,* 2009) and on GF-677 (*P. amygdalus* x *P. persica*) in Iran (Salehi *et al.,* 2011).

The most characteristic symptoms caused by AlmWB on almond trees are i) shoot proliferation on the main trunk with appearance of witches'-broom, ii) development of many auxillary buds on the branches, with small and chlorotic leaves, iii) general decline of the tree, yield losses and final dieback. A total produce loss arises 1-2 years after the initial appearance of the symptoms (Abou-Jawdah *et al.,* 2002). Concerning peach and nectarine trees, the first symptom observed is the early flowering (15 to 20 days earlier than normal), followed by the earlier development of all the buds of the infected branches. In addition, some months after the normal flowering period, phyllody and serrate, slim, light green leaves on the plant branches and witches'-brooms on the trunk and the crown of the trees are present (Abou-Jawdah *et al.,* 2009). Diseases similar to AlmWB, inducing axillary proliferation and little yellow leaves in almond trees were reported in Iran (Verdin *et al.,* 2003; Zirak *et al.,* 2009). Interestingly, grafting experiments and molecular analyses revealed that, up to now, AlmWB does not affect plum (*P. domestica*), apricot (*P. armeniaca*) and cherry (*P. avium*) trees (Abou-Jawdah *et al.,* 2003). Nevertheless, its rapid spread on almond, peach and nectarine orchards confirmed the risk for epidemics in Lebanon and in the other Countries of the Mediterranean area. Phytoplasmas are wall-less parasitic bacteria living exclusively in the plant phloem as consequence of the transmission by sap-sucking insect vectors (Lee *et al.,* 2000). They are classified in '*Candidatus* Phytoplasma' species and in taxonomic group/subgroup according to the sequence of their 16S ribosomal DNA (16SrDNA) (IRPCM, 2004, Zhao *et al.,* 2009). AlmWB is associated with '*Ca.* Phytoplasma phoenicium' strains belonging to taxonomic subgroup 16SrIX-B (Abou-Jawdah *et al.,* 2002; Lee *et al.,* 2012), designated also as 16SrIX-D (Wei *et al.,* 2007; Molino Lova *et al.,* 2011), and its genetic variants (Molino Lova *et al.,* 2011).

The presence and rapid spread of AlmWB in Lebanon entail the activity of one or more vectors. In nature phytoplasmas are mainly transmitted by sap-sucking insects, mainly Hemiptera Auchenorrhyncha (families Cicadellidae and Cixiidae) and Sternorrhyncha (Psillydae) (Weber & Maixner, 1998; Weintraub & Beanland, 2006). Recent study showed that the leafhopper *Asymmetrasca decedens* Paoli plays a major role in spreading the disease within or to nearby stone fruit orchards (Abou-Jawdah *et al.*,, 2014). Moreover, the presence of the disease over distantly located regions, and the detection of AlmWB phytoplasma in other insect species (Dakhil *et al.*,, 2011) may indirectly represent a hypothesis that other potential vectors for AlmWB phytoplasma may be present. Effectively, many phytoplasma diseases (i.e bois noir disease of grapevine) have complex epidemiological cycles involving more than one insect vector and multiple host plants (Maixner, 2011). Since some cixiid species (planthoppers) are known to be vector of phytoplasmas infecting many different crops (Alma *et al.*, 2002; Palermo *et al.*, 2004; Weintraub & Beanland, 2006; Jović *et al.*, 2007; Pinzauti *et al.*, 2008), the present work was focused on the survey of the cixiid-fauna present in almond and nectarine orchards of Lebanon with particular attention on their natural infection by phytoplasmas. Moreover, transmission trials were carried out with specimens belonging to the most abundant genera in order to verify their possible vectoring activity.

MATERIAL AND METHODS

Study area

The field surveys were conducted during the 4-year period 2010-2013 in two AlmWB infested orchards of almond and nectarine trees, and surroundings. The almond 0.2 ha orchard was located in Feghal, district of Jbeil, in the north of Lebanon at about 165m a.s.l. The 72 almond trees were 10-40 years old. The nectarine 0.4 ha orchard was located in Kfarkela, district of Marjayoun, in the south of Lebanon at about 600m a.s.l. The 200 nectarine trees were about 10 years old. In the selected orchards no insecticide treatments were performed during the sampling period.

Insect collection

The investigation was carried out by means of yellow sticky traps and Malaise traps. Only one Malaise trap (165cm x 115cm x 190cm) was installed into each orchard among a group of infected trees in the years

2010-2012. Six double-sided yellow sticky traps (10cm x 30cm) were placed, in each orchard, only during the two-year period 2011-2012 and were uniformly distributed in the centre of the orchards between infected trees. All sticky traps, and the Malaise trap jars, were replaced every two weeks. Ethanol 70% was the preservative liquid used for filling the jars. The insect samplings were carried out from the beginning of February till the end of December in 2010, while in the following two years, in the light of the results obtained in 2010, from the end of March till the end of November. Most of the cixiids collected by means of Malaise and yellow sticky traps were further analysed for phytoplasma presence. Additional direct insect samplings were performed by means of a sweeping net (35cm diam) in spring and late summer 2010 and 2011 and by a hand-held mechanical aspirator (D-Vac Vacuum Insect Net-Model 122, Rincon-Vitova Insectaries, Ventura, CA, USA) in spring 2012 and 2013. These collecting activities were done in the same orchards previously mentioned and their surroundings on different wild plants present in the area. The insects collected in spring 2012 were used for controlled transmission trials and then analysed for phytoplasma presence.

Plant sampling

In the spring time of the years 2010-2013, leaf samples were collected from 15 almond and 10 nectarine plants showing typical AlmWB symptoms such as witches'-broom, phyllody, virescence, and chromatic alterations of the leaves (Abou-Jawdah *et al.,* 2003), and located in the orchard of Feghal and Kfarkela respectively. Moreover, leaf and petiole samples were collected from wild plants where Cixiidae specimens had been captured. In particular samples from 10 and 19 plants of the weed species *Smilax aspera* L., a monocotyledonous plant of the family Smilacaceae, were collected in autumn 2011 and in spring 2012 respectively in the north of Lebanon. In the south, samples from 29 and 11 plants of the weed *Anthemis* sp., a dicotyledonous plant of the family Asteraceae, were collected during spring 2012 and 2013.

Insect identification

Cixiid specimens, after being sorted out from the material caught by the traps, were individually identified with a stereo microscope. The identification at genus level was gained through the external morphological features (Kalkandelen, 1987; Holzinger *et al.*, 2003). For species identification, male genitalia (aedeagus,

parameres and anal tube) were carefully dissected and placed in a 10% potassium hydroxide solution for about one day in order to remove membranous soft tissues and make them semi-diaphanous. They were subsequently observed and preserved immersed in glycerin.

Transmission trials

The insects collected in May 2012 by means of the D-Vac, on the weeds in the orchards and their surroundings, were used for controlled transmission trials. The putative vectors, belonging to different genera, were caged in small batches (1-5 individuals) onto a GF305 potted peach seedlings as indicator plant for phytoplasmas (Gentit *et al.*, 1998, Marcone *et al.*, 2010). Each plant was isolated under a plexiglass squarecross-section cage (28X28X40cm). A total number of 61 specimens belonging to the genera *Cixius*, *Tachycixius*, *Eumecurus* and *Pentastiridius* were isolated on 1, 11, 1 and 1 caged peach plant respectively. In particular, 1 cage containing *Cixius* specimens and 6 cages containing *Tachycixius* specimens were set up with insects collected in the north of Lebanon on *S. aspera*, while 5 cages containing *Tachycixius* specimens, 1 containing *Eumecurus* specimens and 1 containing *Pentastiridius* specimens were set up with insects collected in the south of Lebanon on *Anthemis* sp.

After a 2-4 days inoculation access period, the insects were collected and preserved in 100% ethanol for further morphological identification and molecular analyses for phytoplasma detection. At the end of the trials all the test plants were transferred into an insect-proof greenhouse for monitoring symptom development.

DNA extraction

DNA extraction from insects

Total genomic DNA was extracted from individual planthoppers following a protocol adapted from Marzachì *et al.,* (1998). Briefly, the ethanol-preserved adults were dried onto filter paper and homogenised in a CTAB-based buffer (2% w/v cetyl-trimethyl-ammonium-bromide (CTAB); 1.4 MNaCl; 20 mM EDTA pH 8.0; 100 mMTris-HCl pH 8.0; 0.2% β-mercaptoethanol). After incubation at 60°C for 30 min, DNA was extracted with one volume of chloroform:isoamylalchool 24:1 v/v solution and then precipitated with

the addition of one volume of cold isopropanol. The DNA pellet was then washed with 70% ethanol, vacuum dried and resuspended in 100 µl TE pH 8.0.

DNA extraction from plants

Total DNA was extracted from examined plants using a modified Doyle & Doyle (1990) protocol. Briefly, leaf veins and petioles (0.5g) were separated from the lamina with sterile scalpels, immersed in liquid nitrogen, and ground using sterile pestles and mortars. Pre-warmed CTAB-based buffer (2.5% w/v cetyl-trimethyl-ammonium-bromide (CTAB); 100mM Tris pH8.0, 1.4M NaCl; 50mM EDTA pH8; 1% PVP-40; 0.5% ascorbic acid) were added to the crushed tissues, homogenized by mechanical pestle, and held at 60°C for 20 minutes. After incubation, DNA was extracted by adding iso-amylalcohol:chloroform (1:24) and precipitated by incubation with isopropanol at -20°C for 20 minutes. Nucleic acid pellet was washed with 70% and 80% ethanol, air-dried, suspended in 50 µl of deionized autoclaved water and maintained at -30°C until use.

PCR and sequencing analyses

The identification of phytoplasmas extracted from insects and plants was carried out through direct and nested PCR, using respectively the semi-specific primer pair AlWF2/AlWR2 (Abou-Jawdah *et al.*, 2003) and the universal phytoplasma primer pairs P1/P7 and R16F2n/R16R2 (Gundersen & Lee, 1996). DNAs extracted from phytoplasma strains FegA11-4 ('*Ca*. Phytoplasma phoenicium', subgroup 16SrIX-B), PEY (*Pichris echioides* yellows phytoplasma, subgroup 16SrIX-C), EY1 ('*Ca*. Phytoplasma ulmi', subgroup 16SrV-A), STOL ('*Ca.* P. solani', subgroup 16SrXII-A), and AY1 ('*Ca*. Phytoplasma asteris', subgroup 16SrI-B) were included for comparisons; 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 mixture without DNA template were used as negative controls. Semi-specific AlWF2/AlWR2 PCR reaction consisted of one cycle at 95°C for 2 minutes, 35 cycles at 94°C for 30 seconds, 54°C for 30 seconds and 72°C for 30 seconds, and a final extension step at 72°C for 7 minutes. Nested PCR was performed in order to confirm doubtful results, to improve the possibility of phytoplasma

detection, and to characterise the isolated phytoplasmas. An aliquot of 2 µL of the diluted (1:30) P1/P7 PCR products from the first amplification was used as a template for the nested PCR. Reaction conditions were as in the original papers.

All amplifications were performed with a thermocycler, S1000^{TM} (Bio-Rad, CA, USA) in 20 (insects) or 25 (plants) µL reaction volume in the case of AlWF2/AlWR2 and P1/P7 PCRs and in 50 µL in the case of F2n/R2 PCR, containing 100µM of each of the four dNTPs, 0.5 µM of each primer, 2 mM MgCl2, 1x polymerase buffer, 1 unit *Taq* polymerase [Bioline, MA, USA (insects) or Promega, Milan, Italy (plants)]) and 1-2 µL sample DNA. All the amplification products were analyzed by electrophoresis in 1% agarose gel, followed by staining with ethidium bromide and observed on UV transilluminator. Amplicons from nested PCRs, after purification by GenEluteTM PCR Clean-Up Kit (Sigma-Aldrich, MO, USA) (insects) or by NucleoSpin® Gel and PCR Clean-Up Kit (Macherey-Nagel GmbH & Co., Düren, Germany) (plants), were sequenced to achieve at least 4x coverage per base position. In detail, each PCR product was sequenced by employing primers R16F2n and R16R2, and also two primers (IX-for: 5'-AGTGTCGGGTTTTGGCTCGGTACTG-3'; IX-rev: 5'-TTCCGGATAACGCTCGCCCCTTATG-3'), internal to the F2n/R2 fragment, designed in the present work based on the 16S rDNA nucleotide sequence of the '*Ca*. Phytoplasma phoenicium' reference strain A4 (accession number AF515636). DNA sequencing was performed in an ABI PRISM 377 automated DNA sequencer (Applied Biosystems, Monza, Italy). The nucleotide sequence data were assembled by employing the Contig Assembling program of the sequence analysis software BIOEDIT, version 7.1.9 (http://www.mbio.ncsu.edu/Bioedit/bioedit.html). Sequences were compared with the GenBank database using the software BlastN (http://www.ncbi.nim.nih.gov/BLAST/) with the aim of searching possible identity. Moreover, affiliation of identified phytoplasmas to taxonomic 16Sr group/subgroup was determined by *in silico* RFLP analyses of F2n/R2 amplicons carried out using the software iPhyClassifier (http://plantpathology.ba.ars.usda.gov/cgi-bin/resource/iphyclassifier.cgi, Zhao *et al.*, 2009).

Phylogenetic analysis

Phytoplasma 16S rRNA gene sequences from this study and from GenBank were used to construct phylogenetic trees. Minimum evolution analysis was carried out using the Neighbor-Joining method and

bootstrap replicated 1000 times with the software MEGA5 (http://www.megasoft-ware.net/index.html) (Tamura *et al.*, 2011).

RESULTS

Insect collection and identification

A total of 736 cixiid specimens were collected by means of Malaise and yellow sticky traps during the three-year period 2010-2012, whereof 522 from the Malaise trap and 173 from yellow sticky traps.

In northern Lebanon the Malaise trap collected 65 specimens in 2010, 164 in 2011 and 74 in 2012, while the yellow sticky traps collected 35 specimens in 2011 and 38 in 2012. Down south, the Malaise trap collected 23 specimens in 2010, 32 in 2011 and 169 in 2012, while the yellow sticky traps collected 83 specimens in 2011 and 53 in 2012. The following genera were identified: *Cixius*, *Tachycixius*, *Eumecurus*, *Oliarus*, *Pentastira*, *Pentastiridius* and *Hyalesthes*. Within each genus, except for *Cixius*, *Oliarus* and *Pentastiridius*, more than one species were found out, but, according to the available literature, only for few of them the species level was achieved. Nine different *taxa* were sorted in the genus *Tachycixius*, 5 for *Eumecurus*, 2 within *Pentastira* and *Hyalesthes* genera for a total of 21 *taxa*. Since the specific identification relies mainly on male genitalia, only male specimens were attributed, whereas the females were only named at genus level. Comparing the genitalia morphology to the available literature for Euro-mediterranean and Middle East area, among the 9 *taxa* within the genus *Tachycixius* 6 were identified as *Tachycixius viperinus* Dlabola, *Tachycixius bidentifer* Dlabola, *Tachycixius cypricus* Dlabola, *Tachycixius logvinenkovae* Dlabola, *Tachycixius creticus* Dlabola and *Tachycixius cf remanei* D'Urso (Dlabola, 1965a; Kalkandelen, 1988; D'Urso, 1999). Among the 5 species belonging to the genus *Eumecurus* 2 were identified as *Eumecurus gyaurus* Dlabola and *Eumecurus angustiformis* (Linnaeus) (Kalkandelen, 1989) whereas *Pentastira cf megista* Emeljanov (Kalkandelen, 1993) is the only one determined in the genus *Pentastira*. Concerning the genus *Hyalesthes* the 2 species were determined as *Hyalesthes obsoletus* Signoret and *Hyalesthes hani* Hoch (Hoch & Remane, 1985). As previously mentioned only one *Pentastiridius* species was collected and identified as *Pentastiridius suezensis*-group while within the genus *Oliarus* the specimens were determined as *Oliarus zercanus* Dlabola (Dlabola, 1965b). The unique species

of *Cixius* did not correspond to any species currently known for the cited geographical area therefore it will be indicated as *Cixius* sp. However the definitive taxonomic position of all these species needs further systematic revision to be clarified, nevertheless the mentioned names will be used in this paper to indicate those species. For the sake of simplicity the data will be shown grouping them under genus level. The most abundant genus was *Tachycixius* with 342 specimens all collected by Malaise and yellow sticky traps, followed by *Eumecurus* (173 spec.), *Hyalesthes* (98 spec.) *Cixius* (97 spec.), *Pentastira* (11 spec.) and *Pentastiridius* (4 spec.). During the three years, the genera *Tachycixius, Cixius* and *Hyalesthes* showed to have two flight-peaks, one in spring and one in autumn; on the contrary *Eumecurus* had only one flight-peak in summer (Figs. 1 and 2). The 11 specimens of *Pentastira* were all collected in August, while 3 *Pentastiridius* specimens were collected in August and 1 in October. Concerning the genus *Hyalesthes*, 10 *H. hani* and 3 *H. obsoletus* males were collected between the second half of May and the first half of June, while other 37 *H. obsoletus* males were collected between September and the first half of November. In the north *Tachycixius* was the most abundant genus followed by *Cixius*, while in the south *Eumecurus* was the most abundant genus followed by *Hyalesthes* and *Tachycixius*. A comparison between sticky and Malaise trap captures, being the former six elements per field, shows that *Cixius, Tachycixius* and *Eumecurus*, among the other cixiid genera, were more frequent on the Malaise than on the sticky traps, while *Pentastira* was collected almost in the same quantity with the two sampling methods. On the contrary *Hyalesthes* specimens were more frequent on sticky traps in southern Lebanon. The additional direct samplings were done on the different wild plants observed in the collecting sites (Table 1). No specimens were collected by means of sweeping net neither up north nor down south in 2010 and 2011. On the contrary, in 2012 and 2013, the use of the D-Vac permitted to find cixiids on the weeds but only on the species *S. aspera* in the north and on *Anthemis* sp. in the south, plants commonly spread in those areas. In particular, in 2012, 22 *Tachycixius* and 4 *Cixius* specimens were collected on *S. aspera*, while 18 *Tachycixius*, 5 *Pentastiridius* and 1 *Eumecurus* specimens were sampled on *Anthemis* sp.. In 2013, 4 and 5 *Tachycixius* specimens were collected on *S. aspera* and *Anthemis* sp. respectively. No cixiids were found on the other wild plant species listed in Table 1.

Detection of phytoplasma infections in insects and plants

A total of 451 specimens belonging to the family Cixiidae and collected from yellow sticky traps and the Malaise traps were processed as previously described for phytoplasma detection and identification. Moreover, 52 specimens collected on *S. aspera* and *Anthemis* sp. with the D-Vac were tested. The expected fragment of approximately 390 bp was obtained with the semi-specific primer pair AlWF2/AlWR2 in the four genera *Cixius*, *Tachycixius, Eumecurus* and *Hyalesthes*, while the nested PCR performed with the phytoplasma universal primers R16F2n/R2 allowed to obtain an amplicon of 1200 bp, in the genera *Cixius*, *Tachycixius*, *Eumecurus*, *Pentastiridius* and *Hyalesthes* (Tables 2, 3 and 5). Concerning the insects collected by Malaise and yellow-sticky traps, 7/28, 4/28 and 1/28 males belonging to the genus *Tachycixius* and giving positive signal with the semi-specific primers AlWF2/AlWR2 were previously identified as *T. bidentifer, T. viperinus* and *T. cf creticus* respectively. Moreover, also 1 *T. cf cypricus* and 1 *T. viperinus* collected by means of the D-VAC on *S. aspera* and *Anthemis* sp. respectively as well as 1 *Cixius* sp. collected on *S. aspera* gave the expected amplicon with the primers AlWF2/AlWR2. Primer pairs AlWF2/AlWR2 and R16F2n/R2 primed amplification of DNA from templates derived from all symptomatic almond and peach plants (Table 4). On the other hand, AlWF2/AlWR2 and F2n/R2 primed amplification of DNA from templates derived from 9 and 5 plants of *S. aspera*, respectively. Moreover, AlWF2/AlWR2 and R16F2n/R16R2 primed amplification of DNA from templates derived from 2 plants of *Anthemis* sp.

Molecular identification of phytoplasmas by sequence analyses

BlastN analyses of the fragment R16F2n/R2 evidenced that phytoplasma strains infecting cixiids in Lebanon share best sequence identity (>99.5%) not only with reference strains of the species '*Ca*. Phytoplasma phoenicium' (GenBank accession AF515836), but also with '*Ca*. Phytoplasma asteris' (M30790), '*Ca*. Phytoplasma solani' (AF248959), and '*Ca*. Phytoplasma mali' (AJ542541). Within each species, phytoplasma strains from insects share a sequence identity >99.8%. Based on virtual RFLP patterns (Fig. 3), iPhyClassifier analyses revealed that (i) '*Ca*. Phytoplasma phoenicium' strains belong to the subgroup 16SrIX-B (similarity coefficient >98% in comparison with pattern of subgroup 16SrIX-B reference strain, GenBank accession AF515636); (ii) '*Ca*. Phytoplasma asteris' strains belong to the subgroups 16SrI-B and -L (similarity coefficient >99% in comparison with patterns of subgroup 16SrI-B

and -L reference strains, GenBank accessionsNC005303 and GU223209, respectively); (iii) '*Ca*. Phytoplasma solani' strains belong to the subgroup 16SrXII-A (similarity coefficient >99% in comparison with pattern of subgroup 16SrXII-A reference strain, GenBank accession AAF248959); (iv) '*Ca*. Phytoplasma mali' strain belongs to the subgroup 16SrX-A (similarity coefficient 100% in comparison with pattern of subgroup 16SrX-A reference strain, GenBank accession AJ542541).

Occurrence of phytoplasma species/groups was differentially distributed in the analyzed cixiid species and in the different geographic areas (Tables 2, 3 and 5). In fact, (i) '*Ca*. Phytoplasma phoenicium' (subgroup 16SrIX-B) strains were identified in Feghal in *Cixius* sp. and *Tachycixius* (including *T. bidentifer*, *T. viperinus, T. cf cypricus* and *T. cf creticus*) specimens and in Kfarkela in *T. viperinus* and *Eumecurus* sp.; (ii) '*Ca*. Phytoplasma asteris' (subgroups 16SrI-B and -L) were found in Feghal in *H. obsoletus*, and in specimens of the genera *Cixius*, *Tachycixius* (including *T. viperinus*), *Eumecurus* (including *Eumecurus cf. cyaurus*) and *Pentastiridius* and in Kfarkela in specimens of the genus *Eumecurus* only; (iii) '*Ca*. Phytoplasma solani' (subgroup 16SrXII-A) was identified in *Tachycixius* and *Eumecurus* specimens in Feghal, and in *H. obsoletus* in Kfarkela; (iv) '*Ca*. Phytoplasma mali' (subgroup 16SrX-A) was detected in *Tachycixius* specimens only in Feghal. Nucleotide sequence analyses of R16F2n/R2 fragments from plants highlighted that phytoplasma strains identified in almond, nectarine, *S. aspera*, and *Anthemis* sp. share a sequence identity > 99.8% between them, and >99.6% in comparison with the reference strain of the species '*Ca*. Phytoplasma phoenicium' (AF515836), underlying their membership to such species (Table 4). Moreover, virtual RFLP pattern analyses carried out through the software iPhyClassifier showed that such '*Ca*. Phytoplasma phoenicium' strains share a similarity coefficient of 100% in comparison with subgroup 16SrIX-B reference strain (AF515636) (Fig. 4). 16S rDNA nucleotide sequences from representative phytoplasma strains identified in the present work were deposited at NCBI GenBank database (Table 5).

Phylogenetic analyses clearly showed that phytoplasma strains identified in insects and plants are positioned together within the '*Ca*. Phytoplasma phoenicium' (subgroup 16SrIX-B) cluster. Furthermore, clustering of other phytoplasma strains identified in insects confirmed their affiliation to the species '*Ca*. Phytoplasma asteris' (subgroups 16SrI-B/-L), '*Ca*. Phytoplasma solani' (subgroup 16SrXII-A), and '*Ca*. Phytoplasma mali' (subgroup 16SrX-A).

Transmission trials

Two of the 14 peach plants inoculated with field collected cixiids tested positive for AlmWB phytoplasma AlWF2/AlWR2 PCR. These plants, tested at 6, 12 and 24 months after inoculation, gave PCR positive results only one year after inoculation via insects without showing any symptom yet. The presence of '*Ca.* Phytoplasma phoenicium' in the test plants was then confirmed after 24 months.

Two of the 37 *Tachycixius* analysed at the end of the trials were positive to AlmWB phytoplasma strains (Table 6). These specimens, identified as *T. cf cypricus* and *T. viperinus*, were collected on *S. aspera* and *Anthemis* sp., respectively and were members of the batches that transmitted '*Ca.* Phytoplasma phoenicium' to the test peach plants. Also one of the *Cixius* used in the trials was positive to '*Ca.* Phytoplasma phoenicium', but no positive signal was recorded from the respective plant. No individuals of *Eumecurus* spp. and *Pentastiridius* spp. were positive to AlmWB phytoplasma, but 2 out of the 3 specimens of *Pentastiridius* that gave positive signal with the generic primers R16F2n/R2 were infected with 16SrI-B phytoplasma.

DISCUSSION

Nowadays, the devastating economic impact of almond witches' broom (AlmWB) disease is mostly restricted to the Middle East, but it deserves particular attention as an emerging threat with real risk of introduction in the Mediterranean Basin and Europe. Interestingly, the very rapid spread of AlmWB-associated pathogen, '*Ca.* Phytoplasma phoenicium', over large geographical areas suggests the presence of efficient insect vector(s). Nevertheless, AlmWB is not classified as a quarantine disease yet, probably due to the poor knowledge on its epidemiology and, in particular, on its transmission from plant to plant.

The knowledge of the insect vectors is one of the crucial key for managing a disease and to avoid further spreading to other geographical areas. When nothing or very few is known about insect vectors of a plant pathogen big efforts are required to identify these insects. It is not always easy and different sampling techniques should often be combined, due to the different life cycle of the insects. Recently, the leafhopper *A. decedens* was reported as a vector of AlmWB phytoplasma within or to nearby stone fruit orchards (Abou-Jawdah *et al.*,, 2014). Moreover, the presence of the disease over distantly located regions, and the

detection of AlmWB phytoplasma in other insect species (Dakhil *et al.*,, 2011) represent a hypothesis that other potential vectors for AlmWB phytoplasma may be present. In the present work, we used both yellow sticky and Malaise traps to obtain a great scale collections of cixiids.

Yellow sticky traps are largely used for monitoring some leafhopper species (Cicadellidae) for their effectiveness (Purcell & Elkinton 1980; Power *et al.*, 1992). They are generally considered inefficient in capturing cixiids (Weber & Maixner, 1998; Nicoli Aldini *et al.*, 2003) probably due to a very reduced planthoppers' flight activity and low response to colour, anyhow they allowed us to obtain significant data on the dynamics of some genera. Although the sticky traps placed in each orchard were in number of six instead of one like for Malaise traps, we compare the total specimen number captured by the former taken together with the total number obtained from the latter. Nevertheless, data collected during this survey show how Malaise and sticky traps placed into the two orchards, subject matter of this research, captured almost the same total number of specimens. This occurred for most of the genera found out except for *Tachycixius* and *Eumecurus* which were the most abundant in specimens and collected mostly by Malaise traps both in the north and in the south. This result could be explained by a higher population density for these two genera than the others and lead to think that the Malaise traps were more efficient. Malaise traps are, as previously specified, made up of a large vertical fine net which intercept indiscriminately all flying insects. Its surface is about 10 times the one of the 6 sticky traps combined together. The collections performed in the two years 2011 and 2012 by means of the two trapping methods [Malaise: *Tachycixius* (227), *Eumecurus* (110); Sticky: *Tachycixius* (51), *Eumecurus* (63)] point out that the number of specimens collected by Malaise is not larger than 4.45 times the amount collected with the sticky traps. In light of this data it could be stated that these latter might be considered more efficient. However, the need to obtain a higher number of specimens in good condition for species determination and molecular diagnosis, leads us to consider the Malaise more useful for the purpose of this survey. However, the usefulness of the sticky traps is confirmed for monitoring given species though they do not provide a reliable estimate of field planthopper population density.

The need to capture living specimens for transmission trials pushed us to perform two additional direct sampling methods. The sweepnet, the first one used in the field, did not succeed whilst the D-vac demonstrated to be the most suitable in this case. This result could be explained by the elusive behavior of

the mentioned cixiid taxa, as observed in the field, which seem to prefer mainly to hide among the *Smilax* bushes creepers and the basal stems and leaves, closer to the ground, of *Anthemis*. Since the net edge could not reach the soil surface or penetrate the dense hair of the spiny *Smilax* bushes, the sweeping did not catch the insects in the net. On the contrary, the suction power of the D-vac could catch hidden cixiids even in the deepest part of the vegetation or closer to the ground.

The data obtained by the field surveys make possible some considerations about the life cycle of the collected cixiid genera. *Cixius, Tachycixius and Hyalesthes* were shown to have two flight-peaks, one in spring and one in autumn. This might be related to their feature of accomplishing two generations per year. In Israel it was already demonstrated that *H. obsoletus* is able to accomplish two generations per year, since two separate flight peaks were found out during the monitoring activities, one lasting about two weeks in June and one four weeks in middle September (Klein *et al.*, 2001). Combining this data with the geographical position of Lebanon referred to Israel, and their similar south-mediterranean climate, it is likely to assert that *Cixius* and *Tachycixius* are able to accomplish two generations per year as well. Moreover, we can confirm the bivoltinism of *H. obsoletus* for Lebanon too, while considering the data obtained with *H. hani* it seems that this latter species accomplishes only one generation/year. Unfortunately, only 3 specimens of the genus *Pentastiridius* were collected in August and one in October, therefore it is unlikely to state or venture a hypothesis about its life cycle. On the contrary, throughout the 3-years collecting period, the genus *Eumecurus* showed always one flight-peak in summer between July and August as well as the 11 specimens of *Pentastira* which were collected in August. Based on these data it is possible to hypothesize a monovoltine cycle both for *Eumecurus* and *Pentastira*.

Cixiids are long since considered a very controversial taxon, rich of shortcomings with regard both to the systematic classification of genera and species and their distribution. Many specialists even claim that in some geographical areas, such as the Mediterranean area, there are still many species unknown to science (D'Urso, 1995; Guglielmino & Bückle, 2007). The genus *Tachycixius* Wagner, for example, presently includes 24 species. 21 of them are currently arranged into 5 species-groups, *T. canariensis*-group, *T. viperinus*-group, *T. pyrenaicus*-group, *T desertorum*-group and *T. pilosus*-group, owing to their morphological affinity (Holzinger 2000). This further highlights the need for deep and comprehensive revisions of genera to elucidate the systematic position of taxa belonging to the family Cixiidae. Since the

complexity and difficulty of this task a deepening, also supported by a molecular approach to untangle the cases where morphology and chorology are not sufficient alone, might be useful.

Molecular analyses and preliminary transmission trials gave interesting information on the potential role of these different cixiid genera in the transmission of phytoplasmas in Lebanon. *Tachycixius*, *Cixius*, *Eumecurus* and *Hyalesthes* were demonstrated to be able to acquire '*Ca.* Phytoplasma phoenicium' while the species *T. cf. cypricus* and *T. viperinus* seem to be able to transmit the AlmWB phytoplasma to healthy peach plants. This result should be further verified because the two specimens were members of batches together with other individuals belonging also to different species. Anyhow it was proven that at least the genus *Tachycixius* can transmit '*Ca.* Phytoplasma phoenicium'. Although the only positive specimen of *Cixius* sp. failed to transmit the phytoplasma, we cannot completely exclude the vector activity of this species. This individual died before the end of the inoculation access period and probably the feeding activity on the test plant was not sufficient to transmit the phytoplasma.

Although some of the collected species are already reported for the Middle-East or surrounding areas (Demir *et al.,* 2007), almost nothing is known on their biology. This lack makes transmission trials problematic. Without knowing the host plants during their life cycle, it is quite impossible the setting up of laboratory rearings and completed controlled transmission trials as a consequence. For this reason only field naturally infected specimens were used, but their identification could be done only *a posteriori* after dissection of male genitalia. In the case of conventional transmission trials to healthy test plants using batches of insects it is a big disadvantage. To overcome this problem transmission trials to artificial diet using single individuals should be taken into account for further research.

The field natural infection rate of the genus *Tachycixius* was lower compared with the one recorded for the genus *Cixius* (15.3% vs 52.9% in the north of Lebanon), but the population density in the orchards was considerably higher for the first one, with important outcomes on the disease epidemiology. Interestingly, extended molecular analyses for the '*Ca.* Phytoplasma phoenicium' detection in the collected insects revealed also the presence of other phytoplasmas. '*Ca.* phytoplasma asteris' (subgroups 16SrI-B and -L) was recorded in the genera *Tachycixius, Eumecurus, Pentastiridius* and *Hyalestes*. This phytoplasma has been reported in many herbs and trees in Europe and America, but never in Lebanon (Lee *et al.,* 2004). Anyway, it was largely reported in diverse cultivated host plants in surrounding areas, i.e. in rapeseed,

462 Niger seed, Russian olive, spinach, canola, sugar beet, and sweet cherry in Iran (Salehi *et al.*, 2005, 2011;
463 Rashidi *et al.*, 2010; Tazehkand *et al.*, 2010; Zirak *et al.*, 2010, Vaali *et al.*, 2011), in peach and tomato in
464 Jordan (Anfoka & Fattash, 2003, 2004), in grapevine and in celosia in Israel (Tanne *et al.*, 2000; Orenstein
465 *et al.*, 2001). Moreover, concerning fruit trees the subgroup 16SrI-B was reported in *Pyrus communis* L.,
466 *P. persica* and *P. salicina* Lindl. in Croatia (Križanac *et al.*, 2010). '*Ca.* Phytoplasma asteris' is associated
467 to many insect vectors such as the leafhoppers *Macrosteles* spp., *Euscelis* spp., *Scaphytopius* spp. and
468 *Aphrodes* spp. (Weintraub & Beanland 2006). In Lebanon '*Ca.* Phytoplasma asteris' has been reported
469 infecting the leafhoppers *Euscelis incises* Kirschbaum and *Psammotettix provincialis* Ribaut (Choueiri *et*
470 *al.*, 2007) but it has never been associated to cixiids before. Similarly, it is the first report of the presence
471 of '*Ca*. phytoplasma mali' (subgroup 16SrX-A) in Lebanon and in the genus *Tachycixius*. Although '*Ca.*
472 Phytoplasma mali' is the causal agent of a serious proliferation disease of apple and for this strictly
473 associated with apple plants, it has also been recorded in many other plant species mainly rosaceous ones:
474 e.g. *Crataegus monogyna* Jacq. in Italy (Tedeschi *et al.*, 2009), *P. avium*, *P. armeniaca* and *P. domestica*
475 in Slovenia (Mehle *et al.*, 2007), in *P. domestica* with plum decline symptoms in Tunisia (Ben Khalifa &
476 Fakhfakh, 2011). The finding of this phytoplasma in Lebanon opens new perspective in the study of fruit
477 tree phytoplasmas in this Country in the light also of the recent report of '*Ca.* Phytoplasma mali' in the
478 neighbor Syria (Al-Jabor, 2012). On the contrary '*Ca*. phytoplasma solani' already reported in grapevines
479 and solanaceous plants in Lebanon and in neighboring Countries (Salar *et al.*, 2007; Contaldo *et al.*, 2011;
480 Salem *et al.*, 2013; Zahavi *et al.*, 2013) and in other host plants in Iran (Zirak *et al.*, 2009; Sichani *et al.*,
481 2011) (subgroup 16SrXII-A) is widely spread all over the world and it is known to be transmitted by
482 polyphagous planthoppers of the family Cixiidae (Quaglino *et al.*, 2013) but its association with the genera
483 *Tachycixius* and *Eumecurus* is something new.
484 Such evidences highlighted the large diffusion in Middle East Countries of phytoplasmas carried by several
485 insects identified in the present study. Thus, it is reasonable to investigate more accurately the potential
486 vectoring role of these cixiids for transmitting '*Ca.* Phytoplasma mali', '*Ca.* Phytoplasma asteris' and '*Ca.*
487 Phytoplasma solani'.
488 In the light of the results obtained in the present study, if cixiids will be confirmed to be among the main
489 vectors and considering that they are very often polyphagous (even if monophagous or oligophagous

490 species occur), on herbs, shrubs and/or trees with nymphs living underground and feeding on roots, the role
491 of wild weeds in the epidemiology of the disease seems to be crucial. For these insects, almond and peach
492 could be considered only dead-hosts for the phytoplasma. On the other hand the recent finding concerning
493 the possible role of *A. decedens* as vector of '*Ca.* Phytoplasma phoenicium' (Abou-Jawdah *et al.*,, 2014)
494 could explain the epidemic spread of the AlmWB disease inside almond orchards. To corroborate and
495 confirm this theory, new surveys are required to better understand the real phytoplasma reservoirs and the
496 biological cycle of the vector(s) with special attention to its/their host plants.
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498
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515
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Table 1 Wild plants examined in almond and peach orchards in Feghal and Kfarkela during insect sampling activities.

Species in Feghal	Species in Kfarkela
Allium sp.	*Amaranthus gracilis* Desf.
Amaranthus sp.	*Amaranthus graecizans* L.
Aristolochia sp.	*Amaranthus* sp.
Asparagus sp.	*Anthemis* sp.
Asteraceae sp.	*Asteraceae* sp.
Capparis spinosa L.	*Capparis spinosa* L.
Clematis sp.	*Convolvulus* sp.
Convolvulus sp.	*Cuscuta* sp.
Euphorbia sp.	*Eroclium* sp.
Ficus carica L.	*Erysimum bonannianum* Presl.
Geranium purpureum Vill.	*Euphorbia* sp.
Heliotropium sp.	*Heliotropium* sp.
Hypericum sp.	*Inula viscosa* L.
Inula viscosa L.	*Lactuca serriola* L.
Laurus nobilis L.	*Malus domestica* Borkh.
Malva sylvestris L.	*Malva sylvestris* L.
Olea europaea L.	*Matricaria* sp.
Origanum syriacum L.	*Medicago* sp.
Osyris alba L.	*Neslia apiculata* Fisch.
Papaver sp.	*Olea cuspidata* Wall.
Pistacia palaestina Boiss.	*Olea europaea* L.
Poaceae sp.	*Onobrychis* sp.
Polypodiales sp.	*Ononis* sp.
Quercus sp.	*Poaceae* sp.
Rahia sp.	*Poa* sp.
Rhamnus alaternus L.	*Rhus coriaria* L.
Rhamnus punctata Boiss.	*Rumex acetosella* Koch.
Salvia hierosolymitana Boiss.	*Scolymus maculatus* L.
Smilax aspera L.	*Sinapis arvensis* L.
Solanum nigrum L.	*Senecium* sp.
Solanum sp.	*Solanum* sp.
Spartium junceum L.	*Trifolium* sp.
Teucrium stachyophyllum	*Urospermum* sp.
Trifolium clypeatum L.	
Vitis vinifera L.	

Table 2 Cixiids collected by Malaise and yellow sticky traps in the years 2010-2012 positive with the semi-specific primers AlWF2/AlWR2 and further analysed by nested PCR and sequencing for phytoplasma subgroup affiliation.

Locality	Cixiids	No. of samples tested	ALWF2/ALWR2 PCR positive	F2n/R2 PCR positive	Subgroup affiliation [(a)] 16SrIX-B
Feghal	*Tachycixius* spp.	183	28	9	5
	Cixius sp.	68	36	22	16
	Hyalesthes spp.	4	0	-	-
	Eumecurus spp.	36	2	0	
Kfarkela	*Tachycixius* spp.	40	0	-	-
	Cixius sp.	5	0	-	-
	Hyalesthes spp.	65	1	0	-
	Eumecurus spp.	47	1	1	1
	Pentastira cf. megista	3	0	-	-

[(a)]Based on 16S rDNA sequence identity determined by BlastN, and virtual RFLP similarity coefficient determined by iPhyClassifier

Table 3 Identification and taxonomic determination of other phytoplasmas carried by cixiids collected with Malaise and yellow sticky traps in the years 2010-2012 that were negative with the semi-specific primers in direct PCR.

Locality	Cixiids	No. of samples tested	F2n/R2 PCR positive	Species/subgroup affiliation [(a)]			
				CaPast 16SrI-B	CaPast 16SrI-L	CaPmal 16SrX-A	CaPsol 16SrXII-A
Feghal	*Tachycixius* spp	155	12	5		2	1
	Cixius sp.	32	2	-		-	1
	Hyalesthes spp.	4	1	1	-	-	-
	Eumecurus spp.	34	9	5	2	-	1
Kfarkela	*Tachycixius* spp	40	0	-	-	-	-
	Cixius sp.	5	0	-	-	-	-
	Hyalesthes spp.	64	4	-	-	-	2
	Eumecurus spp.	46	14	8	-	-	-
	Pentastira cf. megista	3	0	-	-	-	-

[(a)] Based on 16S rDNA sequence identity determined by BlastN, and virtual RFLP similarity coefficient determined by iPhyClassifier
CaPast: '*Ca*. Phytoplasma asteris'; CaPmali: '*Ca*. Phytoplasma mali'; CaPsol: '*Ca*. Phytoplasma solani'

719 **Table 4** Identification and taxonomic determination of phytoplasmas infecting stone fruits and weeds
720
721

Locality	Collecting period	Plant	No. of samples tested	ALWF2/ALWR2 PCR positive	F2n/R2 PCR positive	Species/subgroup affiliation [(a)]
Feghal	May 2010	almond	5	5	5	CaPphoe / IX-B
	May 2011	almond	5	5	5	CaPphoe / IX-B
	May 2012	almond	3	3	3	CaPphoe / IX-B
	May 2013	almond	2	2	2	CaPphoe / IX-B
	Autumn 2011	*S. aspera*	10	0	0	nd
	Spring 2012	*S. aspera*	19	9	5	CaPphoe / IX-B
Kfarkela	May 2010	nectarine	3	3	3	CaPphoe / IX-B
	May 2011	nectarine	3	3	3	CaPphoe / IX-B
	May 2012	nectarine	4	4	4	CaPphoe / IX-B
	Spring 2012	*Anthemis sp.*	29	2	2	CaPphoe / IX-B

722 [(a)] Based on 16S rDNA sequence identity determined by BlastN, and virtual RFLP similarity coefficient determined by iPhyClassifier
723 CaPphoe: '*Ca.* phytoplasma phoenicium'
724
725

726

727 **Table 5** GenBank Accession Numbers of 16S rDNA nucleotide sequences amplified from representative
728 phytoplasma strains identified in insects and plants in Lebanese regions.
729

Strain	Host	Species	Subgr.	Acc. No.
R0_221	*Cixius* sp. ♀	'*Ca*. Phytoplasma. phoenicium'	IX-B	KF583767
R11_34	*Cixius* sp. ♀	'*Ca*. Phytoplasma phoenicium'	IX-B	KF583768
R12_29	*Cixius* sp. ♂	'*Ca*. Phytoplasma phoenicium'	IX-B	KF583769
R12_45	*Cixius* sp. ♂	'*Ca*. Phytoplasma phoenicium'	IX-B	KF583770
R12_139	*Eumecurus* sp. ♀	'*Ca*. Phytoplasma phoenicium'	IX-B	KF583771
R12_266	*Tachycixius* sp. ♀	'*Ca*. Phytoplasma phoenicium'	IX-B	KF583772
R13_130	*Tachycixius viperinus* Dlabola ♂	'*Ca*. Phytoplasma phoenicium'	IX-B	KF583773
R12_254	*Tachycixiuscf. bidentifer* Dlabola ♂	'*Ca*. Phytoplasma phoenicium'	IX-B	KF583774
R12_351	*Tachycixiuscf. creticus* Dlabola ♂	'*Ca*. Phytoplasma phoenicium'	IX-B	KF583775
R13_103	*Tachycixius viperinus* Dlabola ♂	'*Ca*. Phytoplasma asteris'	I-B	KF583776
R13_108	*Eumecurus* sp. ♀	'*Ca*. Phytoplasma asteris'	I-B	KF583777
R12_298	*Tachycixius* sp. ♀	'*Ca*. Phytoplasma asteris'	I-B	KF583778
R13_111	*Eumecurusprope gyaurus* (Dlabola)♂	'*Ca*. Phytoplasma asteris'	I-B	KF583779
R13_123	*Hyalesthes obsoletus* Signoret ♂	'*Ca*. Phytoplasma asteris'	I-B	KF583780
R13_139	*Pentastiridius suezensis*-group ♂	'*Ca*. Phytoplasma asteris'	I-B	KF583781
R13_140	*Pentastiridius* sp. ♀	'*Ca*. Phytoplasma asteris'	I-B	KF583782
R13_105	*Eumecurus* sp. ♀	'*Ca*. Phytoplasma asteris'	I-L	KF583783
R13_112	*Eumecurus prope gyaurus* (Dlabola)♂	'*Ca*. Phytoplasma asteris'	I-L	KF583784
R13_72	*Tachycixius* sp. ♂	'*Ca*. Phytoplasma solani'	XII-A	KF583785
R13_34	*Hyalesthes obsoletus* ♂	'*Ca*. Phytoplasma solani'	XII-A	KF583786
R13_69	*Eumecurus* sp. ♀	'*Ca*. Phytoplasma solani'	XII-A	KF583787
R13_43	*Tachycixius* sp. ♀	'*Ca*. Phytoplasma mali'	X-A	KF583788
Smilax10	*Smilax aspera* L.	'*Ca*. Phytoplasma phoenicium'	IX-B	KF583754
Smilax9	*Smilax aspera* L.	'*Ca*. Phytoplasma phoenicium'	IX-B	KF583755
Smilax12	*Smilax aspera* L.	'*Ca*. Phytoplasma phoenicium'	IX-B	KF583756
Smilax13	*Smilax aspera* L.	'*Ca*. Phytoplasma phoenicium'	IX-B	KF583757
Anth1	*Anthemis* sp.	'*Ca*. Phytoplasma phoenicium'	IX-B	KF583765
Anth2	*Anthemis* sp.	'*Ca*. Phytoplasma phoenicium'	IX-B	KF583766
Na201-1	*Prunus dulcis* (Mill.) D.A.Webb	'*Ca*. Phytoplasma phoenicium'	IX-B	KF583758
Na203-1	*Prunus dulcis* (Mill.) D.A.Webb	'*Ca*. Phytoplasma phoenicium'	IX-B	KF583759
Na208-1	*Prunus dulcis* (Mill.) D.A.Webb	'*Ca*. Phytoplasma phoenicium'	IX-B	KF583760
Na235-1	*Prunus dulcis* (Mill.) D.A.Webb	'*Ca*. Phytoplasma phoenicium'	IX-B	KF583761
SN205	*Prunus persica* var. *nucipersica*	'*Ca*. Phytoplasma phoenicium'	IX-B	KF583762
SN206	*Prunus persica* var. *nucipersica*	'*Ca*. Phytoplasma phoenicium'	IX-B	KF583763
SN209	*Prunus persica* var. *nucipersica*	'*Ca*. Phytoplasma phoenicium'	IX-B	KF583764

730

731

732 **Table 6** Transmission trials of '*Ca.* Phytoplasma phoenicium' to potted peach plants using field collected
733 cixiids.
734

Group	Cixiids				Test plant AlmWB-PCR+
	Locality	Genus	No. of insects	AlmWB-PCR+ / tested	
1	North	*Tachycixius*	3	1/3	+
2	North	*Tachycixius*	3	0/2	-
3	North	*Tachycixius*	5	0/5	-
4	North	*Tachycixius*	2	0/1	-
5	North	*Tachycixius*	5	0/5	-
6	North	*Tachycixius*	4	0/4	-
7	North	*Cixius*	4	1/3	-
8	South	*Tachycixius*	4	0/4	-
9	South	*Tachycixius*	2	0/2	-
10	South	*Tachycixius*	6	1/5	+
11	South	*Tachycixius*	2	0/2	-
12	South	*Tachycixius*	4	0/4	-
13	South	*Pentastiridius*	5	0/4	-
14	South	*Eumecurus*	1	0/1	-

735

736

737 **FIGURE LEGENDS**

738

739 **Figure 1.** Flying periods of the genera *Cixius*, *Tachycixius*, *Eumecurus* and *Hyalesthes* collected in
740 northern Lebanon during the years 2011-2012 with the Malaise trap (a) and the yellow sticky traps (b).

741

742 **Figure 2.** Flying periods of the genera *Cixius*, *Tachycixius*, *Eumecurus* and *Hyalesthes* collected in
743 southern Lebanon during the years 2011-2012 with the Malaise trap (a) and the yellow sticky traps
744 (b).

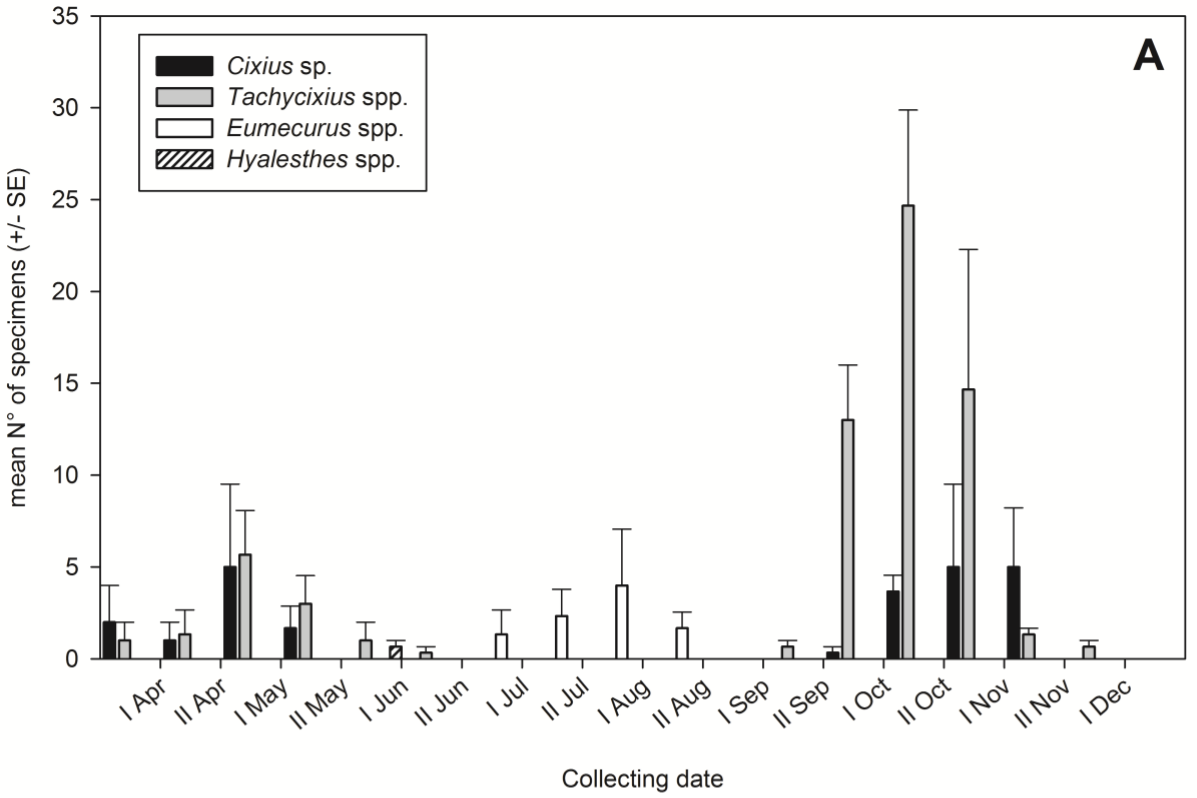
745

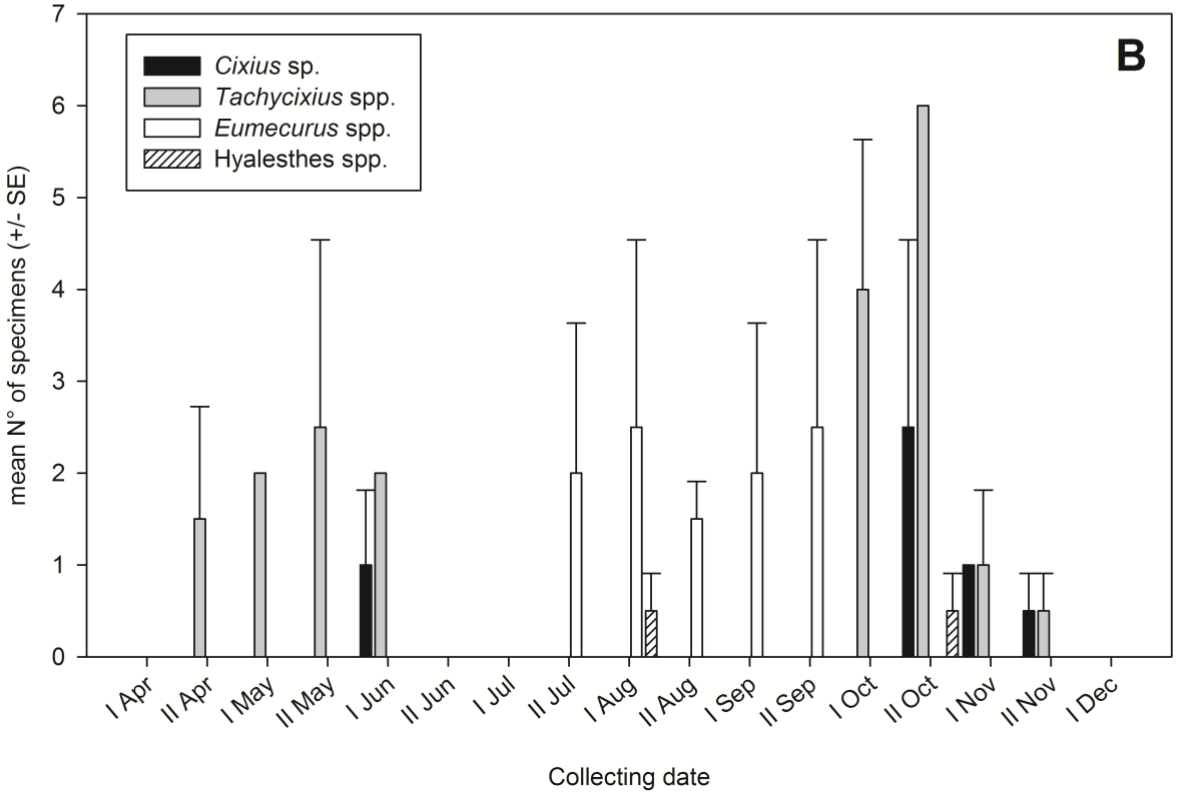
746 **Figure 3.** Collective virtual-RFLP patterns of phytoplasma subgroups 16SrI-B (a), I-L (b), IX-B (c),
747 X-A (d), and XII-A (e), identified in insects and plants in Lebanon.

748

749 **Figure 4.** Phylogenetic tree inferred from analyses of nucleotide sequences of 16S rRNA gene.
750 Minimum evolution analysis was carried out using the neighbor-joining method with the software
751 MEGA4 (36). The reliability of the analyses was subjected to a bootstrap test with 1000 replicates;
752 bootstrap values lower than 60 are not shown. Phytoplasma strains and their nucleotide sequence
753 accession numbers from GenBank are given in the trees. Nucleotide sequences from the present work
754 (Table X) are marked with asterisks. *Acholeplasma palmae* was used for rooting the tree.

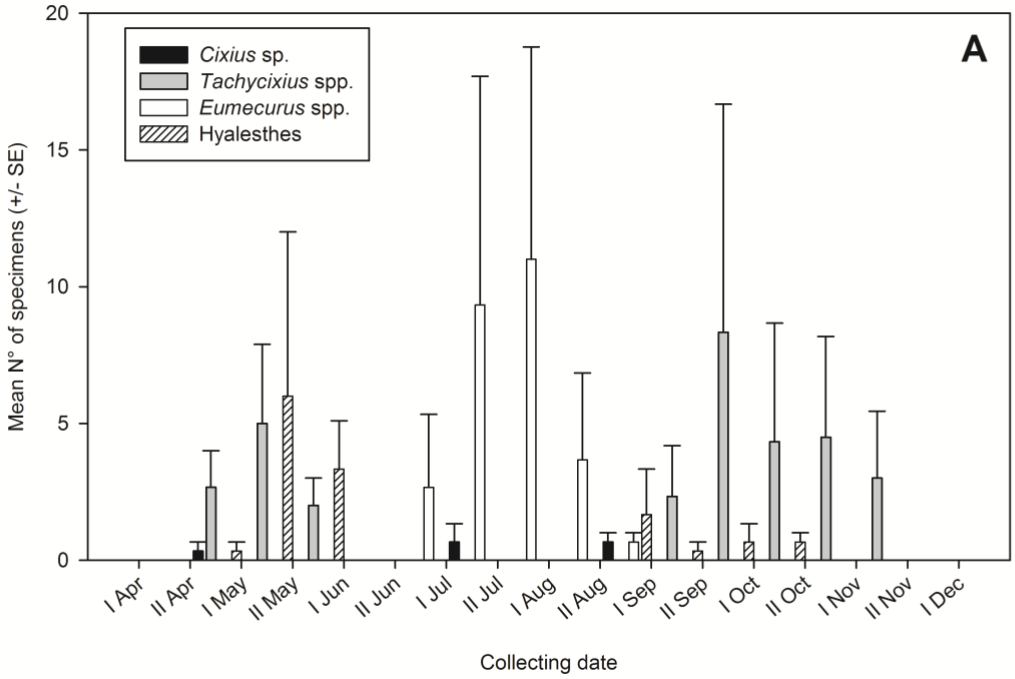
755

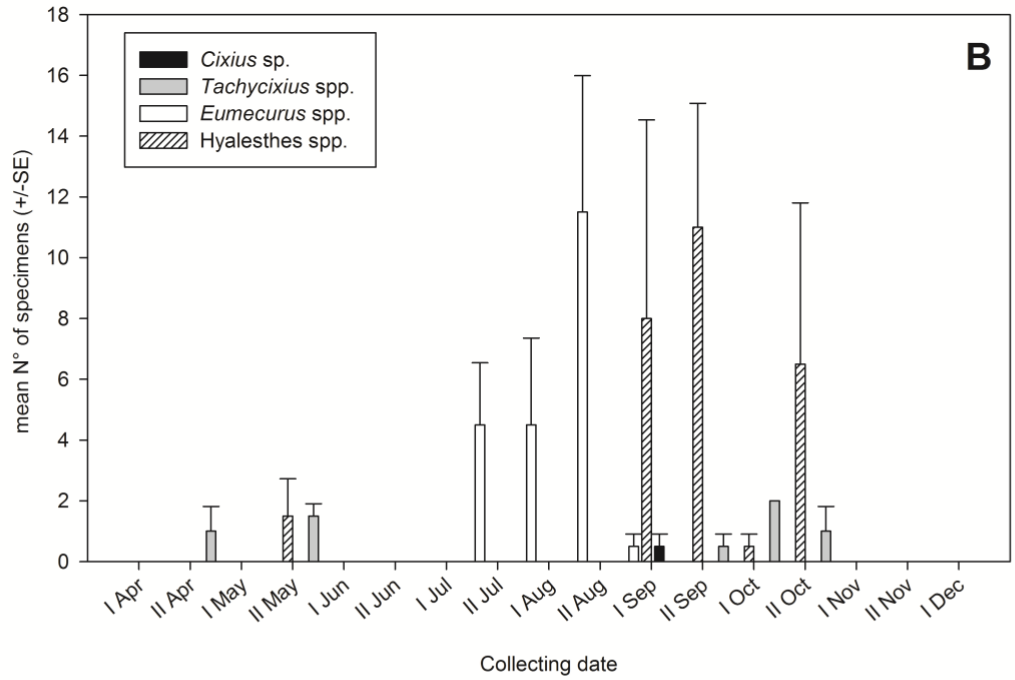

A
Cixius sp.
Tachycixius spp.
Eumecurus spp.
Hyalesthes spp.
mean N° of specimens (+/- SE)
0
5
10
15
20
25
30
35
I Apr
II Apr
I May
II May
I Jun
II Jun
I Jul
II Jul
I Aug
II Aug
I Sep
II Sep
I Oct
II Oct
I Nov
II Nov
I Dec
Collecting date


B
Cixius sp.
Tachycixius spp.
Eumecurus spp.
Hyalesthes spp.
mean N° of specimens (+/- SE)
0
1
2
3
4
5
6
7
I Apr
II Apr
I May
II May
I Jun
II Jun
I Jul
II Jul
I Aug
II Aug
I Sep
II Sep
I Oct
II Oct
I Nov
II Nov
I Dec
Collecting date

756

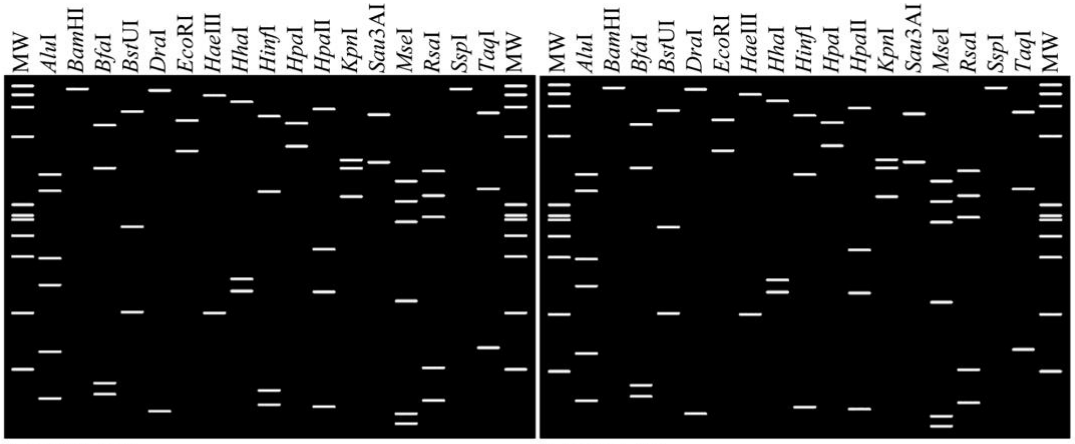
757


A
Cixius sp.
Tachycixius spp.
Eumecurus spp.
Hyalesthes
Mean N° of specimens (+/- SE)
0
5
10
15
20
I Apr
II Apr
I May
II May
I Jun
II Jun
I Jul
II Jul
I Aug
II Aug
I Sep
II Sep
I Oct
II Oct
I Nov
II Nov
I Dec
Collecting date

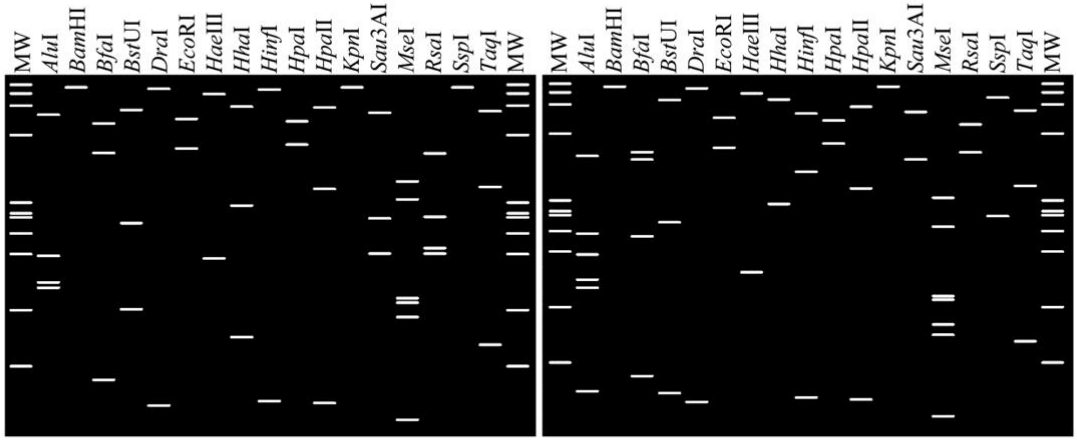

B
Cixius sp.
Tachycixius spp.
Eumecurus spp.
Hyalesthes spp.
mean N° of specimens (+/-SE)
0
2
4
6
8
10
12
14
16
18
I Apr
II Apr
I May
II May
I Jun
II Jun
I Jul
II Jul
I Aug
II Aug
I Sep
II Sep
I Oct
II Oct
I Nov
II Nov
I Dec
Collecting date

758

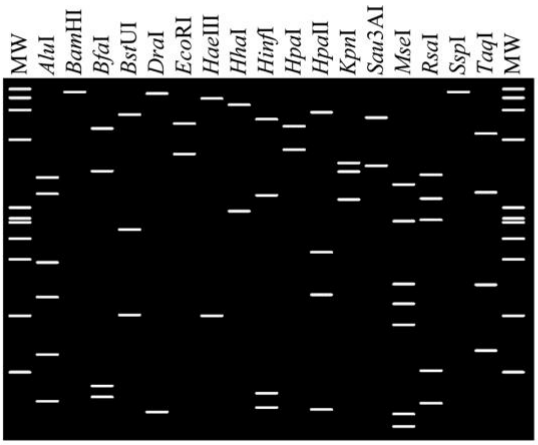
759



(a) 16SrI-B (b) 16SrI-L



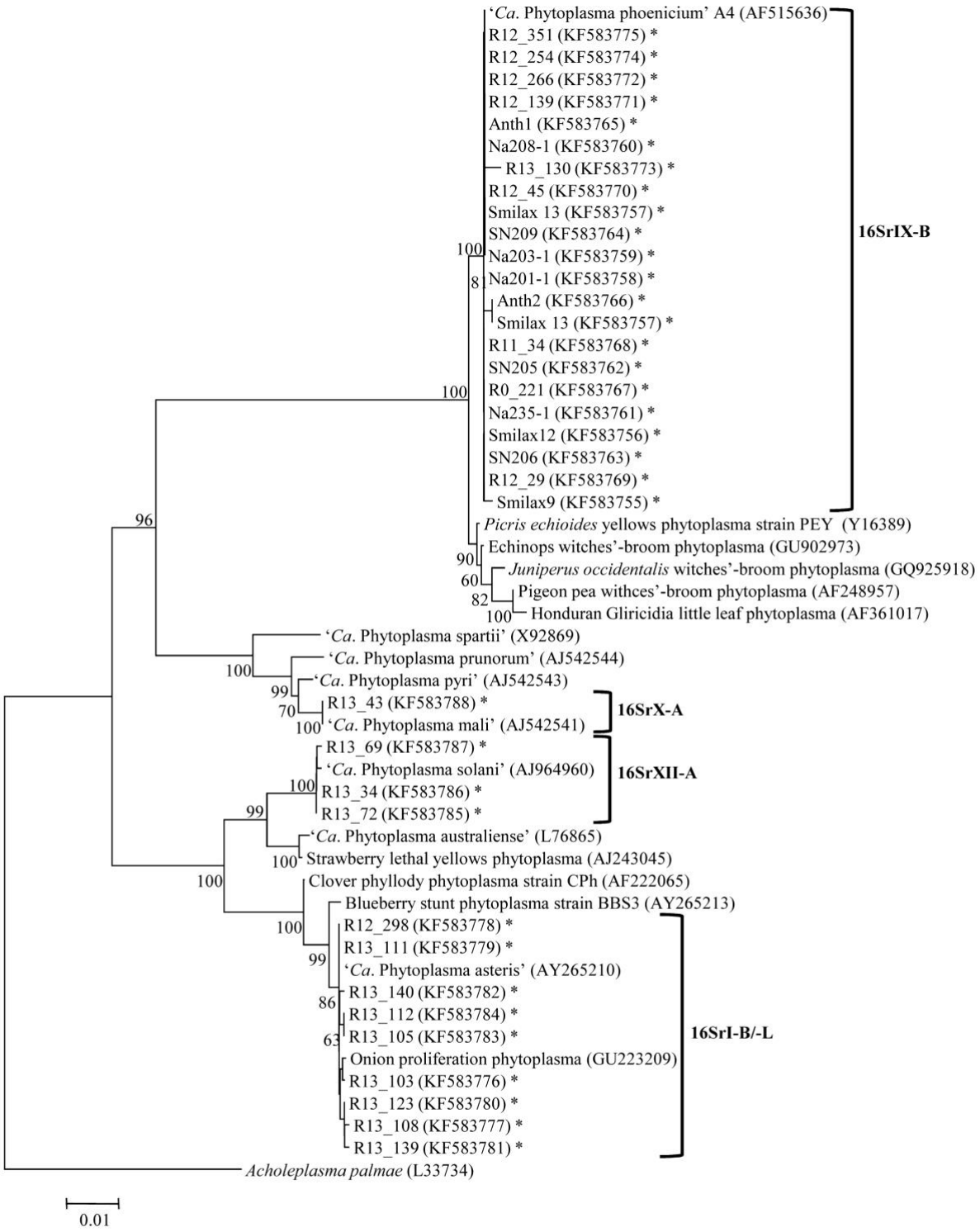
(c) 16SrIX-B (d) 16SrX-A



(e) 16SrXII-A

760

761


'Ca. Phytoplasma phoenicium' A4 (AF515636)
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R12_254 (KF583774) *
R12_266 (KF583772) *
R12_139 (KF583771) *
Anth1 (KF583765) *
Na208-1 (KF583760) *
R13_130 (KF583773) *
R12_45 (KF583770) *
Smilax 13 (KF583757) *
SN209 (KF583764) *
Na203-1 (KF583759) *
Na201-1 (KF583758) *
Anth2 (KF583766) *
Smilax 13 (KF583757) *
R11_34 (KF583768) *
SN205 (KF583762) *
R0_221 (KF583767) *
Na235-1 (KF583761) *
Smilax12 (KF583756) *
SN206 (KF583763) *
R12_29 (KF583769) *
Smilax9 (KF583755) *
16SrIX-B
Picris echioides yellows phytoplasma strain PEY (Y16389)
Echinops witches'-broom phytoplasma (GU902973)
Juniperus occidentalis witches'-broom phytoplasma (GQ925918)
Pigeon pea withces'-broom phytoplasma (AF248957)
Honduran Gliricidia little leaf phytoplasma (AF361017)
'Ca. Phytoplasma spartii' (X92869)
'Ca. Phytoplasma prunorum' (AJ542544)
'Ca. Phytoplasma pyri' (AJ542543)
R13_43 (KF583788) *
'Ca. Phytoplasma mali' (AJ542541)
16SrX-A
R13_69 (KF583787) *
'Ca. Phytoplasma solani' (AJ964960)
R13_34 (KF583786) *
R13_72 (KF583785) *
16SrXII-A
'Ca. Phytoplasma australiense' (L76865)
Strawberry lethal yellows phytoplasma (AJ243045)
Clover phyllody phytoplasma strain CPh (AF222065)
Blueberry stunt phytoplasma strain BBS3 (AY265213)
R12_298 (KF583778) *
R13_111 (KF583779) *
'Ca. Phytoplasma asteris' (AY265210)
R13_140 (KF583782) *
R13_112 (KF583784) *
R13_105 (KF583783) *
Onion proliferation phytoplasma (GU223209)
R13_103 (KF583776) *
R13_123 (KF583780) *
R13_108 (KF583777) *
R13_139 (KF583781) *
16SrI-B/-L
Acholeplasma palmae (L33734)
0.01

762