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

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18	A cixiid survey for natural potential vectors of 'Candidatus
19	Phytoplasma phoenicium' in Lebanon and preliminary transmission
20	trials
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38	Running title: Potential cixiid vectors of 'Ca. Phytoplasma phoenicium'
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43 ABSTRACT

44	Almond witches'-broom (AlmWB) disease, associated with 'Candidatus Phytoplasma phoenicium', is an
45	emerging threat with real risk of introduction in Euro-Mediterranean Countries. Its rapid spread over large
46	geographical areas suggests the presence of efficient insect vector(s). In the present work, a survey on
47	cixiids was carried out in Lebanon in the years 2010-2013 in AlmWB-infested almond and nectarine
48	orchards. Insects were collected by means of different methods, identified with a stereo microscope, and
49	analyzed for phytoplasma identification through 16S rDNA PCR-based amplification and nucleotide
50	sequence analyses. Preliminary transmission trials were performed with the most abundant species.
51	A list of the cixiid genera and species present in the studied area is given as well as some information
52	about their biology. 'Ca. Phytoplasma phoenicium' strains were detected in the genera Cixius,
53	Tachycixius, Eumecurus, and Hyalesthes. Preliminary trials revealed that Tachycixius specimens were
54	able to transmit the detected strains to healthy peach potted seedlings. Further studies are required to
55	better clarify the taxonomic status and the bio-ethology of collected planthoppers and deeply study their
56	role as phytoplasma vectors.
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59	Keywords: almond witches'-broom; planthoppers; Prunus sp.; weeds; 16S rDNA
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71 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).

79 The most characteristic symptoms caused by AlmWB on almond trees are i) shoot proliferation on the 80 main trunk with appearance of witches'-broom, ii) development of many auxillary buds on the branches, 81 with small and chlorotic leaves, iii) general decline of the tree, yield losses and final dieback. A total 82 produce loss arises 1-2 years after the initial appearance of the symptoms (Abou-Jawdah et al., 2002). 83 Concerning peach and nectarine trees, the first symptom observed is the early flowering (15 to 20 days 84 earlier than normal), followed by the earlier development of all the buds of the infected branches. In 85 addition, some months after the normal flowering period, phyllody and serrate, slim, light green leaves on 86 the plant branches and witches'-brooms on the trunk and the crown of the trees are present (Abou-Jawdah 87 et al., 2009). Diseases similar to AlmWB, inducing axillary proliferation and little yellow leaves in 88 almond trees were reported in Iran (Verdin et al., 2003; Zirak et al., 2009). Interestingly, grafting 89 experiments and molecular analyses revealed that, up to now, AlmWB does not affect plum (P. 90 domestica), apricot (P. armeniaca) and cherry (P. avium) trees (Abou-Jawdah et al., 2003). Nevertheless, 91 its rapid spread on almond, peach and nectarine orchards confirmed the risk for epidemics in Lebanon and 92 in the other Countries of the Mediterranean area. Phytoplasmas are wall-less parasitic bacteria living 93 exclusively in the plant phloem as consequence of the transmission by sap-sucking insect vectors (Lee et 94 al., 2000). They are classified in 'Candidatus Phytoplasma' species and in taxonomic group/subgroup 95 according to the sequence of their 16S ribosomal DNA (16SrDNA) (IRPCM, 2004, Zhao et al., 2009). 96 AlmWB is associated with 'Ca. Phytoplasma phoenicium' strains belonging to taxonomic subgroup 97 16SrIX-B (Abou-Jawdah et al., 2002; Lee et al., 2012), designated also as 16SrIX-D (Wei et al., 2007; 98 Molino Lova et al., 2011), and its genetic variants (Molino Lova et al., 2011).

99 The presence and rapid spread of AlmWB in Lebanon entail the activity of one or more vectors. In nature 100 phytoplasmas are mainly transmitted by sap-sucking insects, mainly Hemiptera Auchenorrhyncha 101 (families Cicadellidae and Cixiidae) and Sternorrhyncha (Psillydae) (Weber & Maixner, 1998; Weintraub 102 & Beanland, 2006). Recent study showed that the leafhopper Asymmetrasca decedens Paoli plays a major 103 role in spreading the disease within or to nearby stone fruit orchards (Abou-Jawdah et al., 2014). 104 Moreover, the presence of the disease over distantly located regions, and the detection of AlmWB 105 phytoplasma in other insect species (Dakhil et al., 2011) may indirectly represent a hypothesis that other 106 potential vectors for AlmWB phytoplasma may be present. Effectively, many phytoplasma diseases (i.e 107 bois noir disease of grapevine) have complex epidemiological cycles involving more than one insect 108 vector and multiple host plants (Maixner, 2011). Since some cixiid species (planthoppers) are known to 109 be vector of phytoplasmas infecting many different crops (Alma et al., 2002; Palermo et al., 2004; 110 Weintraub & Beanland, 2006; Jović et al., 2007; Pinzauti et al., 2008), the present work was focused on 111 the survey of the cixiid-fauna present in almond and nectarine orchards of Lebanon with particular 112 attention on their natural infection by phytoplasmas. Moreover, transmission trials were carried out with 113 specimens belonging to the most abundant genera in order to verify their possible vectoring activity.

114

115 MATERIAL AND METHODS

116 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.

123

124 Insect collection

125 The investigation was carried out by means of yellow sticky traps and Malaise traps. Only one Malaise 126 trap (165cm x 115cm x 190cm) was installed into each orchard among a group of infected trees in the 127 years 2010-2012. Six double-sided yellow sticky traps (10cm x 30cm) were placed, in each orchard, only 128 during the two-year period 2011-2012 and were uniformly distributed in the centre of the orchards 129 between infected trees. All sticky traps, and the Malaise trap jars, were replaced every two weeks. 130 Ethanol 70% was the preservative liquid used for filling the jars. The insect samplings were carried out 131 from the beginning of February till the end of December in 2010, while in the following two years, 132 in the light of the results obtained in 2010, from the end of March till the end of November. Most of 133 the cixiids collected by means of Malaise and yellow sticky traps were further analysed for 134 phytoplasma presence. Additional direct insect samplings were performed by means of a sweeping 135 net (35cm diam) in spring and late summer 2010 and 2011 and by a hand-held mechanical 136 aspirator (D-Vac Vacuum Insect Net-Model 122, Rincon-Vitova Insectaries, Ventura, CA, USA) in 137 spring 2012 and 2013. These collecting activities were done in the same orchards previously 138 mentioned and their surroundings on different wild plants present in the area. The insects collected 139 in spring 2012 were used for controlled transmission trials and then analysed for phytoplasma 140 presence.

141

142 Plant sampling

143 In the spring time of the years 2010-2013, leaf samples were collected from 15 almond and 10 nectarine 144 plants showing typical AlmWB symptoms such as witches'-broom, phyllody, virescence, and chromatic 145 alterations of the leaves (Abou-Jawdah et al., 2003), and located in the orchard of Feghal and Kfarkela 146 respectively. Moreover, leaf and petiole samples were collected from wild plants where Cixiidae 147 specimens had been captured. In particular samples from 10 and 19 plants of the weed species Smilax 148 aspera L., a monocotyledonous plant of the family Smilacaceae, were collected in autumn 2011 and in 149 spring 2012 respectively in the north of Lebanon. In the south, samples from 29 and 11 plants of the weed 150 Anthemis sp., a dicotyledonous plant of the family Asteraceae, were collected during spring 2012 and 151 2013.

152

153 Insect identification

154 Cixiid specimens, after being sorted out from the material caught by the traps, were individually 155 identified with a stereo microscope. The identification at genus level was gained through the external 156 morphological features (Kalkandelen, 1987; Holzinger *et al.*, 2003). For species identification, male 157 genitalia (aedeagus, parameres and anal tube) were carefully dissected and placed in a 10% potassium 158 hydroxide solution for about one day in order to remove membranous soft tissues and make them semi-159 diaphanous. They were subsequently observed and preserved immersed in glycerin.

160

161 Transmission trials

162 The insects collected in May 2012 by means of the D-Vac, on the weeds in the orchards and their 163 surroundings, were used for controlled transmission trials. The putative vectors, belonging to different 164 genera, were caged in small batches (1-5 individuals) onto a GF305 potted peach seedlings as indicator 165 plant for phytoplasmas (Gentit et al., 1998, Marcone et al., 2010). Each plant was isolated under a 166 plexiglass squarecross-section cage (28X28X40cm). A total number of 61 specimens belonging to the 167 genera Cixius, Tachycixius, Eumecurus and Pentastiridius were isolated on 1, 11, 1 and 1 caged peach 168 plant respectively. In particular, 1 cage containing Cixius specimens and 6 cages containing Tachycixius 169 specimens were set up with insects collected in the north of Lebanon on S. aspera, while 5 cages 170 containing Tachycixius specimens, 1 containing Eumecurus specimens and 1 containing Pentastiridius 171 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.

176

177 **DNA extraction**

178 **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); 1.4 182 MNaCl; 20 mM EDTA pH 8.0; 100 mMTris-HCl pH 8.0; 0.2% β -mercaptoethanol). After incubation at 183 60°C for 30 min, DNA was extracted with one volume of chloroform:isoamylalchool 24:1 v/v solution 184 and then precipitated with the addition of one volume of cold isopropanol. The DNA pellet was then 185 washed with 70% ethanol, vacuum dried and resuspended in 100 μ l TE pH 8.0.

186

187 **DNA extraction from plants**

188 Total DNA was extracted from examined plants using a modified Doyle & Doyle (1990) protocol. 189 Briefly, leaf veins and petioles (0.5g) were separated from the lamina with sterile scales, immersed in 190 liquid nitrogen, and ground using sterile pestles and mortars. Pre-warmed CTAB-based buffer (2.5% w/v 191 cetyl-trimethyl-ammonium-bromide (CTAB); 100mM Tris pH8.0, 1.4M NaCl; 50mM EDTA pH8; 1% 192 PVP-40; 0.5% ascorbic acid) were added to the crushed tissues, homogenized by mechanical pestle, and 193 held at 60°C for 20 minutes. After incubation, DNA was extracted by adding iso-amylalcohol:chloroform 194 (1:24) and precipitated by incubation with isopropanol at -20°C for 20 minutes. Nucleic acid pellet was 195 washed with 70% and 80% ethanol, air-dried, suspended in 50 µl of deionized autoclaved water and 196 maintained at -30°C until use.

197

198 PCR and sequencing analyses

199 The identification of phytoplasmas extracted from insects and plants was carried out through direct and 200 nested PCR, using respectively the semi-specific primer pair AlWF2/AlWR2 (Abou-Jawdah et al., 2003) 201 and the universal phytoplasma primer pairs P1/P7 and R16F2n/R16R2 (Gundersen & Lee, 1996). DNAs 202 extracted from phytoplasma strains FegA11-4 ('Ca. Phytoplasma phoenicium', subgroup 16SrIX-B), PEY 203 (Pichris echioides yellows phytoplasma, subgroup 16SrIX-C), EY1 ('Ca. Phytoplasma ulmi', subgroup 204 16SrV-A), STOL ('Ca. P. solani', subgroup 16SrXII-A), and AY1 ('Ca. Phytoplasma asteris', subgroup 205 16SrI-B) were included for comparisons; the phytoplasma strains PEY, EY1, STOL, and AY1 were 206 maintained in periwinkle (Catharanthus roseus (L.) G. Don.), while the strain FegA11-4 was identified in 207 AlmWB-diseased almond tree in a previous study (Molino Lova et al., 2011). DNA from healthy 208 periwinkle plants and reaction mixture without DNA template were used as negative controls. Semi-209 specific AlWF2/AlWR2 PCR reaction consisted of one cycle at 95°C for 2 minutes, 35 cycles at 94°C for

210 30 seconds, 54°C for 30 seconds and 72°C for 30 seconds, and a final extension step at 72°C for 7 211 minutes. Nested PCR was performed in order to confirm doubtful results, to improve the possibility of 212 phytoplasma detection, and to characterise the isolated phytoplasmas. An aliquot of 2 μ L of the diluted 213 (1:30) P1/P7 PCR products from the first amplification was used as a template for the nested PCR. 214 Reaction conditions were as in the original papers.

215 All amplifications were performed with a thermocycler, S1000TM (Bio-Rad, CA, USA) in 20 (insects) or 216 25 (plants) µL reaction volume in the case of AlWF2/AlWR2 and P1/P7 PCRs and in 50 µL in the case of 217 F2n/R2 PCR, containing 100µM of each of the four dNTPs, 0.5 µM of each primer, 2 mM MgCl2, 1x 218 polymerase buffer, 1 unit Taq polymerase [Bioline, MA, USA (insects) or Promega, Milan, Italy 219 (plants)]) and 1-2 μ L sample DNA. All the amplification products were analyzed by electrophoresis in 220 1% agarose gel, followed by staining with ethidium bromide and observed on UV transilluminator. 221 Amplicons from nested PCRs, after purification by GenEluteTM PCR Clean-Up Kit (Sigma-Aldrich, 222 MO, USA) (insects) or by NucleoSpin® Gel and PCR Clean-Up Kit (Macherey-Nagel GmbH & Co., 223 Düren, Germany) (plants), were sequenced to achieve at least 4x coverage per base position. In detail, 224 each PCR product was sequenced by employing primers R16F2n and R16R2, and also two primers (IX-225 for: 5'-AGTGTCGGGTTTTGGCTCGGTACTG-3'; IX-rev: 5'-TTCCGGATAACGCTCGCCCCTTATG-226 3'), internal to the F2n/R2 fragment, designed in the present work based on the 16S rDNA nucleotide 227 sequence of the 'Ca. Phytoplasma phoenicium' reference strain A4 (accession number AF515636). DNA 228 sequencing was performed in an ABI PRISM 377 automated DNA sequencer (Applied Biosystems, 229 Monza, Italy). The nucleotide sequence data were assembled by employing the Contig Assembling 230 program of the sequence analysis software BIOEDIT, version 7.1.9 231 (http://www.mbio.ncsu.edu/Bioedit/bioedit.html). Sequences were compared with the GenBank database 232 using the software BlastN (http://www.ncbi.nim.nih.gov/BLAST/) with the aim of searching possible 233 identity. Moreover, affiliation of identified phytoplasmas to taxonomic 16Sr group/subgroup was 234 determined by in silico RFLP analyses of F2n/R2 amplicons carried out using the software iPhyClassifier 235 (http://plantpathology.ba.ars.usda.gov/cgi-bin/resource/iphyclassifier.cgi, Zhao et al., 2009).

236

237 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).

242

243 **RESULTS**

244

245 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.

248 In northern Lebanon the Malaise trap collected 65 specimens in 2010, 164 in 2011 and 74 in 2012, while 249 the yellow sticky traps collected 35 specimens in 2011 and 38 in 2012. Down south, the Malaise trap 250 collected 23 specimens in 2010, 32 in 2011 and 169 in 2012, while the yellow sticky traps collected 83 251 specimens in 2011 and 53 in 2012. The following genera were identified: Cixius, Tachycixius, 252 Eumecurus, Oliarus, Pentastira, Pentastiridius and Hyalesthes. Within each genus, except for Cixius, 253 Oliarus and Pentastiridius, more than one species were found out, but, according to the available 254 literature, only for few of them the species level was achieved. Nine different *taxa* were sorted in the 255 genus Tachycixius, 5 for Eumecurus, 2 within Pentastira and Hyalesthes genera for a total of 21 taxa. 256 Since the specific identification relies mainly on male genitalia, only male specimens were attributed, 257 whereas the females were only named at genus level. Comparing the genitalia morphology to the 258 available literature for Euro-mediterranean and Middle East area, among the 9 taxa within the genus 259 Tachycixius 6 were identified as Tachycixius viperinus Dlabola, Tachycixius bidentifer Dlabola, 260 Tachycixius cypricus Dlabola, Tachycixius logvinenkovae Dlabola, Tachycixius creticus Dlabola and 261 Tachycixius cf remanei D'Urso (Dlabola, 1965a; Kalkandelen, 1988; D'Urso, 1999). Among the 5 species 262 belonging to the genus Eumecurus 2 were identified as Eumecurus gyaurus Dlabola and Eumecurus 263 angustiformis (Linnaeus) (Kalkandelen, 1989) whereas Pentastira cf megista Emeljanov (Kalkandelen, 264 1993) is the only one determined in the genus *Pentastira*. Concerning the genus *Hyalesthes* the 2 species 265 were determined as Hyalesthes obsoletus Signoret and Hyalesthes hani Hoch (Hoch & Remane, 1985).

266 As previously mentioned only one *Pentastiridius* species was collected and identified as *Pentastiridius* 267 suezensis-group while within the genus Oliarus the specimens were determined as Oliarus zercanus 268 Dlabola (Dlabola, 1965b). The unique species of Cixius did not correspond to any species currently 269 known for the cited geographical area therefore it will be indicated as Cixius sp. However the definitive 270 taxonomic position of all these species needs further systematic revision to be clarified, nevertheless the 271 mentioned names will be used in this paper to indicate those species. For the sake of simplicity the data 272 will be shown grouping them under genus level. The most abundant genus was Tachycixius with 342 273 specimens all collected by Malaise and yellow sticky traps, followed by Eumecurus (173 spec.), 274 Hyalesthes (98 spec.) Cixius (97 spec.), Pentastira (11 spec.) and Pentastiridius (4 spec.). During the 275 three years, the genera Tachycixius, Cixius and Hyalesthes showed to have two flight-peaks, one in spring 276 and one in autumn; on the contrary *Eumecurus* had only one flight-peak in summer (Figs. 1 and 2). The 277 11 specimens of *Pentastira* were all collected in August, while 3 *Pentastiridius* specimens were collected 278 in August and 1 in October. Concerning the genus Hyalesthes, 10 H. hani and 3 H. obsoletus males were 279 collected between the second half of May and the first half of June, while other 37 H. obsoletus males 280 were collected between September and the first half of November. In the north Tachycixius was the most 281 abundant genus followed by Cixius, while in the south Eumecurus was the most abundant genus followed 282 by Hyalesthes and Tachycixius. A comparison between sticky and Malaise trap captures, being the former 283 six elements per field, shows that Cixius, Tachycixius and Eumecurus, among the other cixiid genera, 284 were more frequent on the Malaise than on the sticky traps, while *Pentastira* was collected almost in the 285 same quantity with the two sampling methods. On the contrary Hyalesthes specimens were more frequent 286 on sticky traps in southern Lebanon. The additional direct samplings were done on the different wild 287 plants observed in the collecting sites (Table 1). No specimens were collected by means of sweeping net 288 neither up north nor down south in 2010 and 2011. On the contrary, in 2012 and 2013, the use of the D-289 Vac permitted to find cixiids on the weeds but only on the species S. aspera in the north and on Anthemis 290 sp. in the south, plants commonly spread in those areas. In particular, in 2012, 22 Tachycixius and 4 291 Cixius specimens were collected on S. aspera, while 18 Tachycixius, 5 Pentastiridius and 1 Eumecurus 292 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.

295

296 Detection of phytoplasma infections in insects and plants

297 A total of 451 specimens belonging to the family Cixiidae and collected from yellow sticky traps and the 298 Malaise traps were processed as previously described for phytoplasma detection and identification. 299 Moreover, 52 specimens collected on S. aspera and Anthemis sp. with the D-Vac were tested. The 300 expected fragment of approximately 390 bp was obtained with the semi-specific primer pair 301 AlWF2/AlWR2 in the four genera Cixius, Tachycixius, Eumecurus and Hyalesthes, while the nested PCR 302 performed with the phytoplasma universal primers R16F2n/R2 allowed to obtain an amplicon of 1200 bp, 303 in the genera Cixius, Tachycixius, Eumecurus, Pentastiridius and Hyalesthes (Tables 2, 3 and 5). 304 Concerning the insects collected by Malaise and yellow-sticky traps, 7/28, 4/28 and 1/28 males belonging 305 to the genus *Tachycixius* and giving positive signal with the semi-specific primers AlWF2/AlWR2 were 306 previously identified as T. bidentifer, T. viperinus and T. cf creticus respectively. Moreover, also 1 T. cf 307 cypricus and 1 T. viperinus collected by means of the D-VAC on S. aspera and Anthemis sp. respectively 308 as well as 1 Cixius sp. collected on S. aspera gave the expected amplicon with the primers 309 AlWF2/AlWR2. Primer pairs AlWF2/AlWR2 and R16F2n/R2 primed amplification of DNA from 310 templates derived from all symptomatic almond and peach plants (Table 4). On the other hand, 311 AlWF2/AlWR2 and F2n/R2 primed amplification of DNA from templates derived from 9 and 5 plants of 312 S. aspera, respectively. Moreover, AlWF2/AlWR2 and R16F2n/R16R2 primed amplification of DNA 313 from templates derived from 2 plants of Anthemis sp.

314

315 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 321 patterns (Fig. 3), iPhyClassifier analyses revealed that (i) 'Ca. Phytoplasma phoenicium' strains belong to 322 the subgroup 16SrIX-B (similarity coefficient >98% in comparison with pattern of subgroup 16SrIX-B 323 reference strain, GenBank accession AF515636); (ii) 'Ca. Phytoplasma asteris' strains belong to the 324 subgroups 16SrI-B and -L (similarity coefficient >99% in comparison with patterns of subgroup 16SrI-B 325 and -L reference strains, GenBank accessionsNC005303 and GU223209, respectively); (iii) 'Ca. 326 Phytoplasma solani' strains belong to the subgroup 16SrXII-A (similarity coefficient >99% in comparison 327 with pattern of subgroup 16SrXII-A reference strain, GenBank accession AAF248959); (iv) 'Ca. 328 Phytoplasma mali' strain belongs to the subgroup 16SrX-A (similarity coefficient 100% in comparison 329 with pattern of subgroup 16SrX-A reference strain, GenBank accession AJ542541).

330 Occurrence of phytoplasma species/groups was differentially distributed in the analyzed cixiid species 331 and in the different geographic areas (Tables 2, 3 and 5). In fact, (i) 'Ca. Phytoplasma phoenicium' 332 (subgroup 16SrIX-B) strains were identified in Feghal in Cixius sp. and Tachycixius (including T. 333 bidentifer, T. viperinus, T. cf cypricus and T. cf creticus) specimens and in Kfarkela in T. viperinus and 334 Eumecurus sp.; (ii) 'Ca. Phytoplasma asteris' (subgroups 16SrI-B and -L) were found in Feghal in H. 335 obsoletus, and in specimens of the genera Cixius, Tachycixius (including T. viperinus), Eumecurus 336 (including Eumecurus cf. cyaurus) and Pentastiridius and in Kfarkela in specimens of the genus 337 Eumecurus only; (iii) 'Ca. Phytoplasma solani' (subgroup 16SrXII-A) was identified in Tachycixius and 338 Eumecurus specimens in Feghal, and in H. obsoletus in Kfarkela; (iv) 'Ca. Phytoplasma mali' (subgroup 339 16SrX-A) was detected in Tachycixius specimens only in Feghal. Nucleotide sequence analyses of 340 R16F2n/R2 fragments from plants highlighted that phytoplasma strains identified in almond, nectarine, S. 341 aspera, and Anthemis sp. share a sequence identity > 99.8% between them, and >99.6% in comparison 342 with the reference strain of the species 'Ca. Phytoplasma phoenicium' (AF515836), underlying their 343 membership to such species (Table 4). Moreover, virtual RFLP pattern analyses carried out through the 344 software iPhyClassifier showed that such 'Ca. Phytoplasma phoenicium' strains share a similarity 345 coefficient of 100% in comparison with subgroup 16SrIX-B reference strain (AF515636) (Fig. 4). 16S 346 rDNA nucleotide sequences from representative phytoplasma strains identified in the present work were 347 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).

353

354 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.

359 Two of the 37 Tachycixius analysed at the end of the trials were positive to AlmWB phytoplasma strains 360 (Table 6). These specimens, identified as T. cf cypricus and T. viperinus, were collected on S. aspera and 361 Anthemis sp., respectively and were members of the batches that transmitted 'Ca. Phytoplasma 362 phoenicium' to the test peach plants. Also one of the Cixius used in the trials was positive to 'Ca. 363 Phytoplasma phoenicium', but no positive signal was recorded from the respective plant. No individuals 364 of Eumecurus spp. and Pentastiridius spp. were positive to AlmWB phytoplasma, but 2 out of the 3 365 specimens of Pentastiridius that gave positive signal with the generic primers R16F2n/R2 were infected 366 with 16SrI-B phytoplasma.

367

368 **DISCUSSION**

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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 AlmWBassociated 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. 376 The knowledge of the insect vectors is one of the crucial key for managing a disease and to avoid further 377 spreading to other geographical areas. When nothing or very few is known about insect vectors of a plant 378 pathogen big efforts are required to identify these insects. It is not always easy and different sampling 379 techniques should often be combined, due to the different life cycle of the insects. Recently, the 380 leafhopper A. decedens was reported as a vector of AlmWB phytoplasma within or to nearby stone fruit 381 orchards (Abou-Jawdah et al., 2014). Moreover, the presence of the disease over distantly located 382 regions, and the detection of AlmWB phytoplasma in other insect species (Dakhil et al., 2011) represent 383 a hypothesis that other potential vectors for AlmWB phytoplasma may be present. In the present work, 384 we used both yellow sticky and Malaise traps to obtain a great scale collections of cixiids.

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Yellow sticky traps are largely used for monitoring some leafhopper species (Cicadellidae) for their 386 effectiveness (Purcell & Elkinton 1980; Power et al., 1992). They are generally considered inefficient in 387 capturing cixiids (Weber & Maixner, 1998; Nicoli Aldini et al., 2003) probably due to a very reduced 388 planthoppers' flight activity and low response to colour, anyhow they allowed us to obtain significant 389 data on the dynamics of some genera. Although the sticky traps placed in each orchard were in number of 390 six instead of one like for Malaise traps, we compare the total specimen number captured by the former 391 taken together with the total number obtained from the latter. Nevertheless, data collected during this 392 survey show how Malaise and sticky traps placed into the two orchards, subject matter of this research, 393 captured almost the same total number of specimens. This occurred for most of the genera found out 394 except for *Tachycixius* and *Eumecurus* which were the most abundant in specimens and collected mostly 395 by Malaise traps both in the north and in the south. This result could be explained by a higher population 396 density for these two genera than the others and lead to think that the Malaise traps were more efficient. 397 Malaise traps are, as previously specified, made up of a large vertical fine net which intercept 398 indiscriminately all flying insects. Its surface is about 10 times the one of the 6 sticky traps combined 399 together. The collections performed in the two years 2011 and 2012 by means of the two trapping 400 methods [Malaise: Tachycixius (227), Eumecurus (110); Sticky: Tachycixius (51), Eumecurus (63)] point 401 out that the number of specimens collected by Malaise is not larger than 4.45 times the amount collected 402 with the sticky traps. In light of this data it could be stated that these latter might be considered more 403 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.

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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.

415 The data obtained by the field surveys make possible some considerations about the life cycle of the 416 collected cixiid genera. Cixius, Tachycixius and Hyalesthes were shown to have two flight-peaks, one in 417 spring and one in autumn. This might be related to their feature of accomplishing two generations per 418 year. In Israel it was already demonstrated that H. obsoletus is able to accomplish two generations per 419 year, since two separate flight peaks were found out during the monitoring activities, one lasting about 420 two weeks in June and one four weeks in middle September (Klein et al., 2001). Combining this data with 421 the geographical position of Lebanon referred to Israel, and their similar south-mediterranean climate, it 422 is likely to assert that *Cixius* and *Tachycixius* are able to accomplish two generations per year as well. 423 Moreover, we can confirm the bivoltinism of *H. obsoletus* for Lebanon too, while considering the data 424 obtained with *H. hani* it seems that this latter species accomplishes only one generation/year. 425 Unfortunately, only 3 specimens of the genus Pentastiridius were collected in August and one in October, 426 therefore it is unlikely to state or venture a hypothesis about its life cycle. On the contrary, throughout the 427 3-years collecting period, the genus *Eumecurus* showed always one flight-peak in summer between July 428 and August as well as the 11 specimens of *Pentastira* which were collected in August. Based on these 429 data it is possible to hypothesize a monovoltine cycle both for *Eumecurus* and *Pentastira*.

430 Cixiids are long since considered a very controversial taxon, rich of shortcomings with regard both to the431 systematic classification of genera and species and their distribution. Many specialists even claim that in

432 some geographical areas, such as the Mediterranean area, there are still many species unknown to science 433 (D'Urso, 1995; Guglielmino & Bückle, 2007). The genus Tachycixius Wagner, for example, presently 434 includes 24 species. 21 of them are currently arranged into 5 species-groups, T. canariensis-group, T. 435 viperinus-group, T. pyrenaicus-group, T desertorum-group and T. pilosus-group, owing to their 436 morphological affinity (Holzinger 2000). This further highlights the need for deep and comprehensive 437 revisions of genera to elucidate the systematic position of taxa belonging to the family Cixiidae. Since the 438 complexity and difficulty of this task a deepening, also supported by a molecular approach to untangle the 439 cases where morphology and chorology are not sufficient alone, might be useful.

440 Molecular analyses and preliminary transmission trials gave interesting information on the potential role 441 of these different cixiid genera in the transmission of phytoplasmas in Lebanon. Tachycixius, Cixius, 442 *Eumecurus* and *Hyalesthes* were demonstrated to be able to acquire '*Ca*. Phytoplasma phoenicium' while 443 the species T. cf. cypricus and T. viperinus seem to be able to transmit the AlmWB phytoplasma to 444 healthy peach plants. This result should be further verified because the two specimens were members of 445 batches together with other individuals belonging also to different species. Anyhow it was proven that at 446 least the genus Tachycixius can transmit 'Ca. Phytoplasma phoenicium'. Although the only positive 447 specimen of *Cixius* sp. failed to transmit the phytoplasma, we cannot completely exclude the vector 448 activity of this species. This individual died before the end of the inoculation access period and probably 449 the feeding activity on the test plant was not sufficient to transmit the phytoplasma.

450 Although some of the collected species are already reported for the Middle-East or surrounding areas 451 (Demir et al., 2007), almost nothing is known on their biology. This lack makes transmission trials 452 problematic. Without knowing the host plants during their life cycle, it is quite impossible the setting up 453 of laboratory rearings and completed controlled transmission trials as a consequence. For this reason only 454 field naturally infected specimens were used, but their identification could be done only a posteriori after 455 dissection of male genitalia. In the case of conventional transmission trials to healthy test plants using 456 batches of insects it is a big disadvantage. To overcome this problem transmission trials to artificial diet 457 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 460 was considerably higher for the first one, with important outcomes on the disease epidemiology. 461 Interestingly, extended molecular analyses for the 'Ca. Phytoplasma phoenicium' detection in the 462 collected insects revealed also the presence of other phytoplasmas. 'Ca. phytoplasma asteris' (subgroups 463 16SrI-B and -L) was recorded in the genera Tachycixius, Eumecurus, Pentastiridius and Hyalestes. This 464 phytoplasma has been reported in many herbs and trees in Europe and America, but never in Lebanon 465 (Lee et al., 2004). Anyway, it was largely reported in diverse cultivated host plants in surrounding areas, 466 i.e. in rapeseed, Niger seed, Russian olive, spinach, canola, sugar beet, and sweet cherry in Iran (Salehi et 467 al., 2005, 2011; Rashidi et al., 2010; Tazehkand et al., 2010; Zirak et al., 2010, Vaali et al., 2011), in 468 peach and tomato in Jordan (Anfoka & Fattash, 2003, 2004), in grapevine and in celosia in Israel (Tanne 469 et al., 2000; Orenstein et al., 2001). Moreover, concerning fruit trees the subgroup 16SrI-B was reported 470 in Pyrus communis L., P. persica and P. salicina Lindl. in Croatia (Križanac et al., 2010). 'Ca. 471 Phytoplasma asteris' is associated to many insect vectors such as the leafhoppers Macrosteles spp., Euscelis spp., Scaphytopius spp. and Aphrodes spp. (Weintraub & Beanland 2006). In Lebanon 'Ca. 472 Phytoplasma asteris' has been reported infecting the leafhoppers Euscelis incises Kirschbaum and 473 474 Psammotettix provincialis Ribaut (Choueiri et al., 2007) but it has never been associated to cixiids before. 475 Similarly, it is the first report of the presence of 'Ca. phytoplasma mali' (subgroup 16SrX-A) in Lebanon 476 and in the genus Tachycixius. Although 'Ca. Phytoplasma mali' is the causal agent of a serious 477 proliferation disease of apple and for this strictly associated with apple plants, it has also been recorded in 478 many other plant species mainly rosaceous ones: e.g. Crataegus monogyna Jacq. in Italy (Tedeschi et al., 479 2009), P. avium, P. armeniaca and P. domestica in Slovenia (Mehle et al., 2007), in P. domestica with 480 plum decline symptoms in Tunisia (Ben Khalifa & Fakhfakh, 2011). The finding of this phytoplasma in 481 Lebanon opens new perspective in the study of fruit tree phytoplasmas in this Country in the light also of 482 the recent report of 'Ca. Phytoplasma mali' in the neighbor Syria (Al-Jabor, 2012). On the contrary 'Ca. 483 phytoplasma solani' already reported in grapevines and solanaceous plants in Lebanon and in neighboring 484 Countries (Salar et al., 2007; Contaldo et al., 2011; Salem et al., 2013; Zahavi et al., 2013) and in other 485 host plants in Iran (Zirak et al., 2009; Sichani et al., 2011) (subgroup 16SrXII-A) is widely spread all 486 over the world and it is known to be transmitted by polyphagous planthoppers of the family Cixiidae 487 (Quaglino *et al.*, 2013) but its association with the genera *Tachycixius* and *Eumecurus* is something new.

Such evidences highlighted the large diffusion in Middle East Countries of phytoplasmas carried by several insects identified in the present study. Thus, it is reasonable to investigate more accurately the potential vectoring role of these cixiids for transmitting '*Ca*. Phytoplasma mali', '*Ca*. Phytoplasma asteris' and '*Ca*. Phytoplasma solani'.

492 In the light of the results obtained in the present study, if cixiids will be confirmed to be among the main 493 vectors and considering that they are very often polyphagous (even if monophagous or oligophagous 494 species occur), on herbs, shrubs and/or trees with nymphs living underground and feeding on roots, the 495 role of wild weeds in the epidemiology of the disease seems to be crucial. For these insects, almond and 496 peach could be considered only dead-hosts for the phytoplasma. On the other hand the recent finding 497 concerning the possible role of A. decedens as vector of 'Ca. Phytoplasma phoenicium' (Abou-Jawdah et 498 al., 2014) could explain the epidemic spread of the AlmWB disease inside almond orchards. To 499 corroborate and confirm this theory, new surveys are required to better understand the real phytoplasma 500 reservoirs and the biological cycle of the vector(s) with special attention to its/their host plants.

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Table 1 Wild plants examined in almond and peach orchards in Feghal and Kfarkela during insect sampling activities.

 709

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.	<i>Cuscuta</i> sp.
<i>Euphorbia</i> sp.	Eroclium sp.
Ficus carica L.	Erysimum bonannianum Presl.
Geranium purpureum Vill.	Euphorbia sp.
<i>Heliotropium</i> sp.	Heliotropium sp.
Hypericum sp.	Inula viscosa L.
Inula viscosa L.	<i>Lactuca serriola</i> L.
Laurus nobilis L.	Malus domestica Borkh.
Malva sylvestris L.	Malva sylvestris L.
Olea europaea L.	Matricaria sp.
Origanum syriacum L.	<i>Medicago</i> sp.
<i>Osyris alba</i> L.	Neslia apiculata Fisch.
<i>Papaver</i> sp.	Olea cuspidata Wall.
Pistacia palaestina Boiss.	Olea europaea L.
<i>Poaceae</i> sp.	Onobrychis sp.
Polypodiales sp.	Ononis sp.
Quercus sp.	<i>Poaceae</i> sp.
<i>Rahia</i> sp.	Poa sp.
<i>Rhamnus alaternus</i> L.	<i>Rhus coriaria</i> L.
Rhamnus punctata Boiss.	Rumex acetosella Koch.
Salvia hierosolymitana Boiss.	Scolymus maculatus L.
<i>Smilax aspera</i> 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 a 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.

Hyalesthes spp. *Eumecurus* spp.

Pentastira cf. megista

Kfarkela Tachycixius spp.

Cixius sp.

(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 samj tested	No. of samples	F2n/R2	Species/subgroup affiliation ^(a)			
		tested	PCR positive	CaPast	CaPast	CaPmal	CaPsol
				16SrI-B	16SrI-L	16SrX-A	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	-	-	-	-

723 (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'

Table 4	Table 4 Identification and taxonomic determination of phytoplasmas infecting stone fruits and weeds						
Locality	Collecting period	Plant	No. of samples tested	ALWF2/ALWR2 PCR positive	F2n/R2 PCR positive	Species/subgroup affiliation ^(a)	

2	01		tested	PCR positive	PCR positive	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

728 ^(a) Based on 16S rDNA sequence identity determined by BlastN, and virtual RFLP similarity coefficient determined by iPhyClassifier 729 ^(a) CaPphoe: '*Ca.* phytoplasma phoenicium'

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

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7	3	5

Strain	Host	Species	Subgr.	Acc. No.
R0_221	<i>Cixius</i> sp. \bigcirc	'Ca. Phytoplasma. phoenicium'	IX-B	KF583767
R11_34	Cixius sp. \bigcirc	'Ca. Phytoplasma phoenicium'	IX-B	KF583768
R12_29	Cixius sp. $eean circle a cir$	'Ca. Phytoplasma phoenicium'	IX-B	KF583769
R12_45	Cixius sp. $eean circle a cir$	'Ca. Phytoplasma phoenicium'	IX-B	KF583770
R12_139	<i>Eumecurus</i> sp. \bigcirc	'Ca. Phytoplasma phoenicium'	IX-B	KF583771
R12_266	<i>Tachycixius</i> sp. \bigcirc	'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	<i>Eumecurus</i> sp. \bigcirc	'Ca. Phytoplasma asteris'	I-B	KF583777
R12_298	<i>Tachycixius</i> sp. Q	'Ca. Phytoplasma asteris'	I-B	KF583778
R13_111	<i>Eumecurusprope gyaurus</i> (Dlabola)♂	'Ca. Phytoplasma asteris'	I-B	KF583779
R13_123	Hyalesthes obsoletus Signoret $\stackrel{\wedge}{\supset}$	'Ca. Phytoplasma asteris'	I-B	KF583780
R13_139	Pentastiridius suezensis-group 🖒	'Ca. Phytoplasma asteris'	I-B	KF583781
R13_140	Pentastiridius sp. \bigcirc	'Ca. Phytoplasma asteris'	I-B	KF583782
R13_105	<i>Eumecurus</i> sp. \bigcirc	'Ca. Phytoplasma asteris'	I-L	KF583783
R13_112	<i>Eumecurus prope gyaurus</i> (Dlabola)∂	'Ca. Phytoplasma asteris'	I-L	KF583784
R13_72	<i>Tachycixius</i> sp. δ	'Ca. Phytoplasma solani'	XII-A	KF583785
R13_34	Hyalesthes obsoletus $\stackrel{\wedge}{\supset}$	'Ca. Phytoplasma solani'	XII-A	KF583786
R13_69	<i>Eumecurus</i> sp. \bigcirc	'Ca. Phytoplasma solani'	XII-A	KF583787
R13_43	<i>Tachycixius</i> sp. Q	'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

739 740 Table 6 Transmission trials of 'Ca. Phytoplasma phoenicium' to potted peach plants using field collected cixiids.

Group		Test plant			
	Locality Genus No. o		No. of insects	AlmWB-PCR+ / tested	AlmWB-PCR+
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	-

FIGURE LEGENDS

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745 Figure 1. Flying periods of the genera Cixius, Tachycixius, Eumecurus and Hyalesthes collected in 746 northern Lebanon during the years 2011-2012 with the Malaise trap (a) and the yellow sticky traps 747 (b). 748 749 Figure 2. Flying periods of the genera Cixius, Tachycixius, Eumecurus and Hyalesthes collected in 750 southern Lebanon during the years 2011-2012 with the Malaise trap (a) and the yellow sticky traps 751 (b). 752 753 Figure 3. Collective virtual-RFLP patterns of phytoplasma subgroups 16SrI-B (a), I-L (b), IX-B (c), 754 X-A (d), and XII-A (e), identified in insects and plants in Lebanon. 755 756 Figure 4. Phylogenetic tree inferred from analyses of nucleotide sequences of 16S rRNA gene. 757 Minimum evolution analysis was carried out using the neighbor-joining method with the software 758 MEGA4 (36). The reliability of the analyses was subjected to a bootstrap test with 1000 replicates; 759 bootstrap values lower than 60 are not shown. Phytoplasma strains and their nucleotide sequence 760 accession numbers from GenBank are given in the trees. Nucleotide sequences from the present 761 work (Table X) are marked with asterisks. Acholeplasma palmae was used for rooting the tree.



Collecting date





(a) 16SrI-B

(b) 16SrI-L



(c) 16SrIX-B

(d) 16SrX-A



(e) 16SrXII-A

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