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Original article (Orijinal araştırma)

Determination of potential insect vectors and subgroups of aster yellows phytoplasma in the carrot (*Daucus carota* L.) (Apiaceae) cultivation areas of Ankara and Konya Provinces, Türkiye[1]

Ankara ve Konya (Türkiye) illeri havuç (*Daucus carota* L.) (Apiaceae) ekim alanlarında aster yellows fitoplazmasının altgruplarının ve potansiyel böcek vektörlerinin belirlenmesi

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Abstract

Aster yellows phytoplasma (16Sr-I, AYp) is a widespread plant pathogen affecting a wide range of economically important crops. AYp can be distributed widely via insect vectors and is associated with severe redness and yellowing in carrot leaves. The presence of potential insect vectors of aster yellows phytoplasma was investigated in the Ankara and Konya Provinces, the largest carrot production areas in Türkiye. Forty-five insect samples were collected during the field studies between March and September 2020. Morphological and molecular studies have shown that *Neophilaenus campestris* (Fallén, 1805) (Hemiptera: Aphrophoridae), *Empoasca decipiens* Paoli, 1930 (Hemiptera: Cicadellidae) and *Psammotettix striatus* (L., 1758) (Hemiptera: Cicadellidae) carried 16SrI-F phytoplasma. *Psammotettix striatus* collected from different locations contained subgroup 16SrI-R, as well. In addition, subgroup 16SrI-B was determined in *Cicadula divaricata* Ribaut, 1952 (Hemiptera: Cicadellidae) and in a psyllid (Psylloidea: Psyllidae) species. *Empoasca* sp., *Anaceratagallia* sp. (Hemiptera: Cicadellidae) and *Psammotettix confinis* (Dahlbom, 1850) (Hemiptera: Cicadellidae) were determined as potential phytoplasma vectors. Phytoplasma 16Sr rRNA and insect cytochrome oxidase gene nucleotide sequences were used for phylogenetic analysis. The results will contribute to vector-based control of aster yellows phytoplasmas in carrot cultivation areas.

Keywords: Aster yellows, carrot, insect vector, PCR, phylogenetic

Öz

Aster yellows fitoplazma (AYp), ekonomik açıdan önemli farklı tarım ürünlerini etkileyen yaygın bir bitki patojenidir. AYp, böcek vektörleri aracılığıyla geniş alanlara yayılabilir ve havuç yapraklarında şiddetli kızarıklık ve sararma ile ilişkilendirilir. Türkiye'nin en büyük havuç üretim alanları olan Ankara ve Konya illerinde aster yellows fitoplazmasının potansiyel böcek vektörlerinin varlığı araştırılmıştır. Mart-Eylül 2020 tarihleri arasında arazi çalışmalarında 45 böcek örneği toplanmıştır. Morfolojik ve moleküler çalışmalar *Neophilaenus campestris* (Fallén, 1805) (Hemiptera: Aphrophoridae), *Empoasca decipiens* Paoli, 1930 (Hemiptera: Cicadellidae) ve *Psammotettix striatus* (L., 1758) (Hemiptera: Cicadellidae) türlerinin 16SrI-F altgrubu ile bulaşık olduğunu göstermiştir. Bununla birlikte, farklı lokasyonlardan toplanan *P. striatus* türü ise 16SrI-R altgrubu ile bulaşıktı. *Cicadula divaricata* Ribaut, 1952 (Hemiptera: Cicadellidae) ve bir psyllid (Psylloidea: Psyllidae) türünde 16SrI-B altgrubu saptanmıştır. *Empoasca* sp., *Anaceratagallia* sp. (Hemiptera: Cicadellidae) ve *Psammotettix confinis* (Dahlbom, 1850) (Hemiptera: Cicadellidae) ise potansiyel fitoplazma vektörleri olarak belirlenmiştir. Elde edilen fitoplazma 16S rRNA ve böcek sitokrom oksidaz genlerinin nükleotit dizileri filogenetik çalışmalarda kullanılmıştır. Bu sonuçlar, havuç ekim alanlarında aster yellows fitoplazmalarının vektör kontrolüne katkıda bulunacaktır.

Anahtar sözcükler: Aster yellows, havuç, böcek vektör, PCR, filogenetik

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Introduction

The carrot (*Daucus carota* L.), a member of the family Apiaceae, has a nearly 5000-year history of domestication and continues to be widely produced and consumed (Stolarczyk & Janick, 2011). The largest carrot cultivation areas in Türkiye are the Ankara and Konya Provinces with 578 kt of production annually (TUIK, 2021).

Carrot cultivation has been adversely affected by several phytopathogens and agricultural pests resulting in decreased yield quality and quantity. Among the phytopathogens, phytoplasma strains, *Candidatus Liberibacter solanacearum* Liefting et al. (Bacteria: Phyllobacteriaceae), and *Spiroplasma citri* Saglio et al. (Bacteria: Mycoplasmataceae), in particular, have been associated with significant carrot yield losses as a result of single or mixed infections (Lee et al., 2006; Cebrián et al., 2010; Satta et al., 2017). In addition to the transmission through seeds (Bertolini et al., 2015; Alfaro-Fernández et al., 2017; Carminati et al., 2019; Randa-Zelyüt et al., 2022), insect vectors are also responsible for the spread of phytoplasmas.

Phytoplasmas, Gram-positive bacteria, require host plant cells and insect vectors to maintain their unique life cycles and replication (Hogenhout & Loria, 2008). Effective pathogen-host-vector interactions can have a critical impact on plants, causing significant symptoms and yield losses in their hosts (Hogenhout et al., 2008). These mollicutes, which are found in almost every region of the world and infect more than a thousand plant species, lack a cell wall and are transmitted and spread by phloem insects of the order Hemiptera (Weintraub & Beanland, 2006; Harrison et al., 2014). More specifically, phytoplasmas are mainly spread by insects of the families Cicadellidae and Psyllidae and the superfamily Fulgoroidea, which feed on the phloem sap of infected plants. Therefore, the host range depends on the feeding habits of the insect vectors (Bertaccini, 2007).

Phytoplasmas have been reported to infect various vegetable crops in 47 countries throughout five continents (Kumari et al., 2019). Among them, aster yellows (16SrI) phytoplasmas are the most common across all genera, followed by the peanut witches' broom (16SrII), clover proliferation (16SrVI), and stolbur (16SrXII-A) phytoplasmas (Kumari et al., 2019). Moreover, phytoplasmas belonging to diverse subgroups of the aster yellows (AY) phytoplasma group (16SrI) have recently been related to diseases in carrots, including red leaves, shoot growth, and poor tap root quality (Duduk et al., 2007). In nature, phloem-feeding leafhoppers (Hemiptera: Cicadellidae) and planthoppers transmit AY group phytoplasmas persistently (Hemiptera: Cixiidae) (Weintraub & Beanland, 2006).

Since there is no effective control option directly against phytoplasmas during the cultivation period, the determination of potential insect vectors is necessary to design robust control programs. Therefore, in the present study, potential insect vectors of phytoplasmas collected from carrot cultivation areas in the Ankara and Konya Provinces of Türkiye have been morphologically and molecularly identified. In addition, the phytoplasma groups and subgroups contained by these insect vectors have been determined molecularly and phylogenetic tree and computer-simulated PCR-RFLP analyses were performed.

Materials and Methods

Sampling potential insect vectors

Field surveys were undertaken in carrot fields to collect insects in the Ankara and Konya Provinces of Türkiye in March-September in 2020. Only the areas that have carrot plants showing phytoplasma symptoms such as severe reddening and yellowing were sampled. The collected insects were directly transferred to 96% ethanol and stored at -20°C until used.

Morphological identification

All specimens were gently separated to avoid damaging key morphological characters for accurate identification. Morphological identification of potential vector insects was performed by Prof. Dr. Emine Demir-Özden under a stereo zoom microscope according to Ribaut (1952), Dlabola (1957), Emeljanov (1964), Ossiannilsson (1981) and Holzinger et al. (2003).

DNA isolation and PCR amplifications

Genomic DNA was extracted from insect specimens individually (n = 27) and three individuals as pooled samples (a total of six pools) using Qiagen DNeasy Blood & Tissue Kit following the manufacturer's instructions. Purity, concentration, and quality controls of the extracted DNAs were measured using a spectrophotometer (Nano-Drop 2000, ThermoFisher Scientific, Waltham, MA, USA). Then, DNA extracts were stored at −20°C until used in PCR amplification.

Molecular identification has been performed to verify/support the morphological identification and also to determine the insect species that cannot be identified morphologically due to damaged insect bodies.

The mitochondrial cytochrome oxidase I gene (*COI*) was used to identify insect specimens. *COI* has been known to provide species-level identification and is thus widely used across the animal kingdom (Hebert et al., 2003). Using the universal *COI* primers HCO2198-(5'-TAAACTTCAGGGTGACCAAAAAATCA-3') and LCO1490-(5'-GGTCAACAAATCATAAAGATATTGG-3') designed by Folmer et al. (1994), PCR amplifications were performed in a total reaction volume of 25 µl including 1.25 µl of Taq DNA polymerase (5 U/µl) (Ampliqon, Denmark) and 100-150 ng/µl of DNA. PCR conditions were according to İnak et al. (2021).

A nested PCR was performed to investigate the presence of phytoplasmas in potential insect vectors. The first step of nested-PCR was conducted using the 16S rRNA gene region amplifying P1-(5'-AAGAATTTGATCCTGGCTCAGGATT-3') /P7-(5'-CGTCCTTCATCGGCTCTT-3') universal primers (Deng & Hiruki, 1991). Before being used as a template in the second step of PCR reactions, the PCR products obtained from the first step were diluted at 1:30 with nuclease-free water. In the second step, universal primer pair R16F2n-(5'-GAAACGACTGCTAAGACTGG-3') -R2-(5'-TGACGGGCGGTGTGTACAAACCCCG-3') (Gundersen & Lee, 1996) or fU5-(5'-CGGCAATGGAGGAAACT-3') -rU3-(5'-TTCAGCTACTCTTTGTAACA-3') (Lorenz et al., 1995) were used. The nested-PCR condition was performed as described by Gundersan & Lee (1996). PCR products were separated on a 1% agarose gel with 100 V for 45 minutes.

The nested PCR reactions were performed using 30-50 ng/µl of genomic DNA (or 1 µl of diluted PCR product for the second step), 2.5 µl of 10X PCR buffer, 0.25 µl of 25 mM MgCl2, 1 µl of 10 mM dNTPs, 1 µl of 10 mM of each primer, and 1.25 U of Taq DNA polymerase (5 U/µl) (Ampliqon, Denmark) in a total reaction volume of 25 µl.

Sequencing and phylogenetic analysis

PCR products of the 16S rRNA gene of phytoplasmas and *COI* gene regions of insects obtained through molecular amplification were sequenced bidirectionally (BMlabsis, Ankara, Türkiye). The quality of sequence chromatographs was manually checked using BioEdit v7.0.5 (Hall, 1999).

BLAST analyses were performed to validate the identification of insects. The sequences were submitted to NCBI (National Center for Biotechnology Information). In addition, the similarity ratios of the nucleotide sequences belonging to phytoplasmas were obtained using F2n/R2 primer pair, and their subgroup classifications were determined using the *i*PhyClassifier software (Zhao et al., 2013).

Phylogenetic analyses were performed using the sequences herein obtained and some retrieved from the public GenBank to reveal the positioning of insect *COI* and phytoplasma 16SrRNA genes. All the sequences were aligned using MAFFT (Katoh et al., 2019) and trimmed using BioEdit v7.0.5 (Hall, 1999).

Next, a neighbor-joining phylogenetic tree has been constructed using MEGA X (Kumar et al., 2018) with 1000 bootstraps. The Tamura-3 (T92) (Tamura, 1992) parameter model has been identified to be the best-fit substitution model by MEGA X (Kumar et al., 2018). *Spiroplasma citri* (accession no AM157769) was used as an outgroup.

PCR-RFLP analysis

Restriction fragment length polymorphism (RFLP) analyses were performed using endonuclease enzymes to determine the genetic profiles of phytoplasma PCR products obtained with the F2n/R2 primer pair. The PCR products were digested with 6 U of restriction endonuclease *Alu*I and *Taq*I enzymes separately (Eurx, Estonia). The digested products were separated on a 1.7% agarose gel with 80 V for 2 h. The agarose gel was treated with ethidium bromide and visualized on a UV transilluminator (Genegenius, England).

Computer-simulated PCR-RFLP analyses for nucleotide sequences obtained from amplicons amplified with the F2n/R2 primer pair were visualized with the *i*PhyClassifier software using *Mse*I and *Alu*I endonuclease enzymes (https://plantpathology.ba.ars.usda.gov; Zhao et al., 2013).

Results

Field surveys and morphological identification of insects

During the field surveys, symptoms such as severe reddening and yellowing which were previously associated with phytoplasma infections were observed in the carrot plants. In addition, the roots of plants exhibiting the leaf symptoms also had lateral root development and abnormal discoloration. A total of forty-five potential vector insect specimens were collected from the fields showing these symptoms.

According to morphological investigations, one species *Neophilaenus campestris* (Fallén, 1805) belonging to the Aphrophoridae family, one species belonging to the family Psyllidae, one species *Javesella* sp. of the Delphacidae family, and nine species *Macropsis* sp., *Psammotettix striatus* (L., 1758), *Empoasca* sp., *Psammotettix* sp., *Euscelis incisus* (Kirschbaum, 1858), *Anaceratagallia ribauti* (Ossiannilsson, 1938), *Empoasca decipiens* Paoli, 1930, *Anaceratagallia* sp. and *Cicadula divaricata* (Ribaut, 1952) in the family Cicadellidae were identified (Figure 1 and Table 1). A list of identified insect species is presented in Table 1.

Table 1. Aster yellows phytoplasma potential insect vectors collected from carrot fields

Region	Species	Specimen	Family
Ankara	*Neophilaenus campestris*	2♀ 1♂	Aphrophoridae
	Macropsis sp.	2 ♀	Cicadellidae
	Psammotettix striatus	2♀ 1♂	Cicadellidae
	-	1*	Psyllidae
	Empoasca sp.	6*	Cicadellidae
	Psammotettix sp.	7*	Cicadellidae
Konya	*Euscelis incisus*	1♀	Cicadellidae
	Anaceratagallia ribauti	2♀	Cicadellidae
	Anaceratagallia sp.	3*	Cicadellidae
	Javesella sp.	1♀	Delphacidae
	Psammotettix striatus	9♀ 1♂	Cicadellidae
	Cicadula divaricata	1♀	Cicadellidae
	Empoasca decipiens	2♂;3♀	Cicadellidae
Total		45	

* Individuals that cannot be fully characterized morphologically.

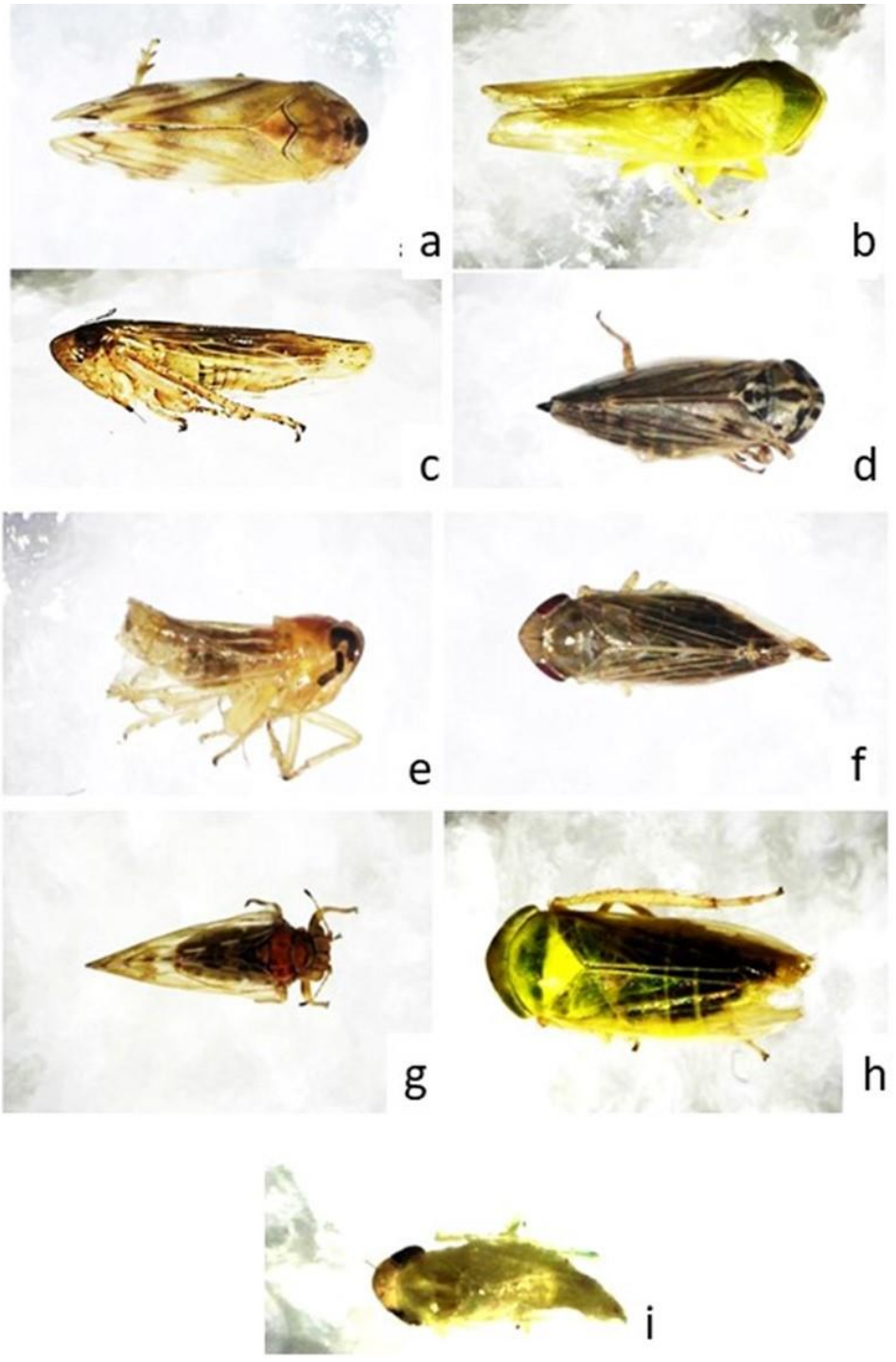

Figure 1. Aster yellows phytoplasma potential vectors collected in carrot fields: a) *Neophilaenus campestris* (Fallen); 1♀ (Aphrophoridae), b) *Macropsis* sp.; 1♀ (Cicadellidae), c) *Euscelis incisus* (Kirschbaum); 1♀ (Cicadellidae), d) *Anaceratagallia ribauti* (Ossiannilsson); 1♀ (Cicadellidae), e) *Javesella* sp.; 1♀ (Delphacidae), f) *Psammotettix striatus*; 1♀ (Cicadellidae), g) Psyllidae species, h) *Cicadula divaricata* (Ribautu); 1♀ (Cicadellidae), and i) *Empoasca decipiens* (Paoli) (Cicadellidae).

Molecular identification of insect species and associated phytoplasmas

For the molecular identification of potential phytoplasma vectors, *COI* sequences from 10 specimens in three species were obtained (accessions: MZ519869-MZ519878). Also, the *COI* gene sequence of the individual belonging to the family Psyllidae could not be obtained, despite the *COI* gene sequences of the morphologically unidentified *Psammotettix* sp., *Anaceratagallia* spp. and *Empoasca* spp. samples being obtained. Thus, all sequences obtained supported the morphological identification to genus level. Unfortunately, we were not able to identify these specimens to species level morphologically. However, BLAST analysis showed that B3, B4, K, B7 and 22B (*Psammotettix* spp.) specimens had 98.48-99.70% identity with deposited sequences of *Psammotettix confinis* from Canada and Ukraine, with accession numbers KR573169 and MW301811, respectively. Therefore, these specimens were considered to be *P. confinis* based on the high similarity of sequences. Although 7B and 9B (*Anaceratagallia* spp*.)* specimens were 96.80% similar to *Anaceratagallia ribauti* species (accession: MK188546), *Anaceratagallia* sequences herein obtained did not been clustered with *A. ribauti* in the phylogenetic tree, suggesting that they were different species. Finally, Y1, Y2 and B1 (*Empoasca* spp.) specimens were found to be 99.84% similar to *Empoasca* sp. from Pakistan (accession: HQ990703), however, the phylogenetic tree showed that they were an *Empoasca* species rather than *E. decipiens* (Table 2).

Table 2. Accession of insect specimens and similarity rates with the GenBank isolates

Insect specimen	Accession	NCBI similarity-Accession Number-Definition
B4 (*Psammotettix* spp.)	MZ519870	99.01%-KR573169-Canada-*Psammotettix confinis*
B1 (*Empoasca* spp.)	MZ519878	99.84%-HQ990703-Pakistan-*Empoasca* sp.
B7 (Psammotettix spp.)	MZ519872	99.00%-KR573169-Canada-*Psammotettix confinis*
K (*Psammotettix* spp.)	MZ519869	98.81%-MW301811-Ukraine-*Psammotettix confinis*-Rub-1
Y1 (*Empoasca* spp.)	MZ519876	99.85%-HQ990703-Pakistan-*Empoasca* sp. HOP-00013
Y2 (*Empoasca* spp.)	MZ519877	99.85%-HQ990703-Pakistan-*Empoasca* sp.
9B (*Anaceratagallia* spp.)	MZ519875	96.80%-MK188546-France-*Anaceratagallia ribauti*
7B (*Anaceratagallia* spp.)	MZ519874	96.80%-MK188546-France-*Anaceratagallia ribauti*
22B *(Psammotettix* spp.)	MZ519871	98.48%-KR573169-Canada-*Psammotettix confinis*
B3 *(Psammotettix* spp.)	MZ519873	99.70%-KR573169-Canada-*Psammotettix confinis*

The 16S rRNA gene region was amplified to determine the presence of phytoplasmas in the DNAs extracted from potential insect vectors. Overall results showing the presence of phytoplasmas in various vector species are given in Table 3. The presence of phytoplasmas was detected in nine of 27 individuals, indicating that 33.3% of screened insects were infected by various subgroups of phytoplasmas. In addition to individual testing, the presence of phytoplasmas was also investigated from pooled insect DNAs and the results showed that three of six pools were positive for phytoplasmas. This result showed that 50% of collective individuals could potentially transmit the pathogen.

All phytoplasma isolates from insect samples (based on either 883 bp or 1.2 kb) were sequenced for further analyses. Three sequences from 12 phytoplasma isolates [1.2 kb (B4-phy, B6-phy, B7-phy, K-phy and 15B-phy), 883 bp (12B-phy, 19B-phy, 21B-phy, 22B-phy, 5B-phy, B1-phy and B2-phy)] were submitted to the GenBank as accessions: MZ457919, MZ464025-MZ464031, MZ450789-MZ450792. The potential vector species and associated phytoplasma species are presented in Table 3. Isolates having 1.2 kb (B4-phy, B7-phy and K-phy isolates) sequences shared 99.92% nt identity with the NCBI isolate M30790 and the 16SrI-F *i*PhyClassifier isolate AY265211. Also, the 15B-phy isolate had 99.92% nt similarity with the NCBI isolate with accession number MN877914 and the 16SrI-B *i*PhyClassifier isolate with accession number AP006628. The B6-phy isolate; the NCBI Iran isolate with accession number MK307856; and the 16SrI-R *i*PhyClassifier isolate with accession number HM067754 had 99.20% nt identity. NCBI data was

used for nt similarity of seven other phytoplasma-infected insect isolates (883 bp). The B1-phy, B2-phy, 5B-phy, 22B-phy, 21B-phy, and 19B-phy isolates had 99.64-99.46% similarity with the Iranian isolate, Bajgah periwinkle little leaf phytoplasma, accession DQ266089. Finally, the 12B-phy isolate had 99.81% nt similarity with the rapeseed phyllody (16SrI-B) Polish isolate accession CP055264.

Table 3. Number of potential vector insects (single/collective) and number of infected specimens

Specimens	Total individuals	Infected/Individual sample	Infected/pooled sample*	16Sr group
Neophilaenus campestris	3	**(5B)** 1/3	-	16SrI **(5B-phy)**
Macropsis sp.	2	0/2	-	-
Euscelis incisus	1	0/1	-	-
Anaceratagallia ribauti	2	0/2	-	-
Javesella sp.	1	0/1	-	-
Psammotettix striatus	13	**(B6) (19B) (22B)** 3/7	0/2	16SrI-R **(B6-phy)**/16SrI **(19B-phy) (22B-phy)**
Psyllidae	1	**(12B)** 1/1	-	16SrI **(12B-phy)**
Empoasca decipiens	5	**(21B)** 1/2	0/1	16SrI **(21B-phy)**
Cicadula divaricata	1	**(15B)** 1/1	-	16SrI-B **(15B-phy)**
Anaceratagallia sp.	3	0/3	-	-
Psammotettix sp.	7	**(B7)** 1/1	**(B4) (K)** 2/2	16SrI-F **(B7-phy) (B4-phy) (K-phy)**
Empoasca sp.	6	**(B1)** 1/3	**(B2)** 1/1	16SrI **(B1-phy) (B2-phy)**
Total	45	9/27	3/6	

* Three individuals were used for each pool.

Phylogenetic analysis

The phylogenetic tree based on *COI* sequences of hemipteran species is presented in Figure 2. In general, the tree showed great resolution to genus level and allowed the genus-level identification of *Empoasca* and *Anaceratagallia* species. Also, the tree, together with BLAST analysis, suggest that the specimens which could not be identified to species level morphologically were *P. confinis* (Dahlbom, 1850), and it was supported with a confidence ratio of 100.

The phylogenetic tree was divided into several evolutionary lineage branches containing 16SrV, 16SrI, 16SrX and 16SrXII groups of phytoplasmas. All of the phytoplasma isolates obtained from potential vector insects were grouped within the 16SrI (aster yellows) main branch and the node of this cluster was supported by a confidence ratio of 99. Major cluster of 16SrI was subdivided into 16SrI-A, 16SrI-B, 16SrI-C, 16SrI-E, 16SrI-F and 16SrI-R subgroups. B1-phy (*Empoasca* sp.), B2-phy (*Empoasca* sp.), 5B-phy (*N. campestris*), 22B-phy (*P. striatus*), 21B-phy (*E. decipiens*), 19B-phy (*P. striatus*), B4-phy (*Psammotettix* sp.), B7-phy (*Psammotettix* sp.) and K-phy (*Psammotettix* sp.) isolates clustered with 16SrI-F subgroup isolates; B6-phy (*P. striatus*) isolate was within 16SrI-R subgroup; 15B-phy (*C. divaricata*) and 12B-phy (Psyllidae species) isolates clustered with 16SrI-B subgroup isolates. Although isolates with both 1.2 kb and 883 bp length sequences were located in a subcluster, these clusters were not supported by high bootstrap values (Figure 3). Moreover, it provided hypothetical information on the main groups which isolates B1-phy, B2-phy, 5B-phy, 22B-phy, 21B-phy, 19B-phy and 12B-phy included. To obtain this hypothetical information, it was necessary to include three or more large phytoplasma groups in the data set.

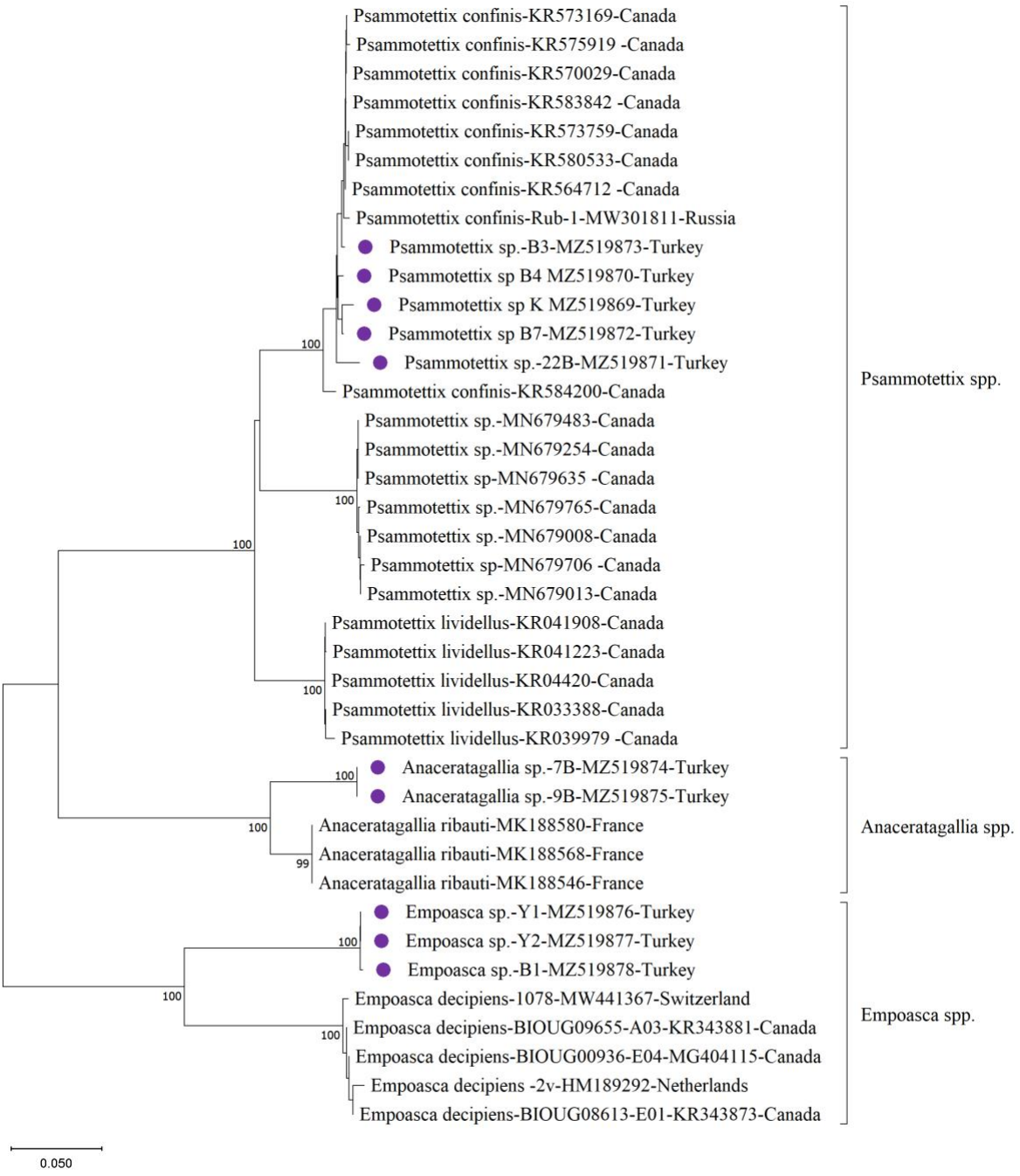

Figure 2. Phylogenetic tree generated by the Neighbor-Joining statistical method, and the substitution model Tamura-3 parameter (T92+G) of nt sequences of the insect *COI* genes. Insect specimens in this study are marked with circle symbols. Bootstrap values on each branch were supported by 1000 replicates; only values greater than 90% were shown.

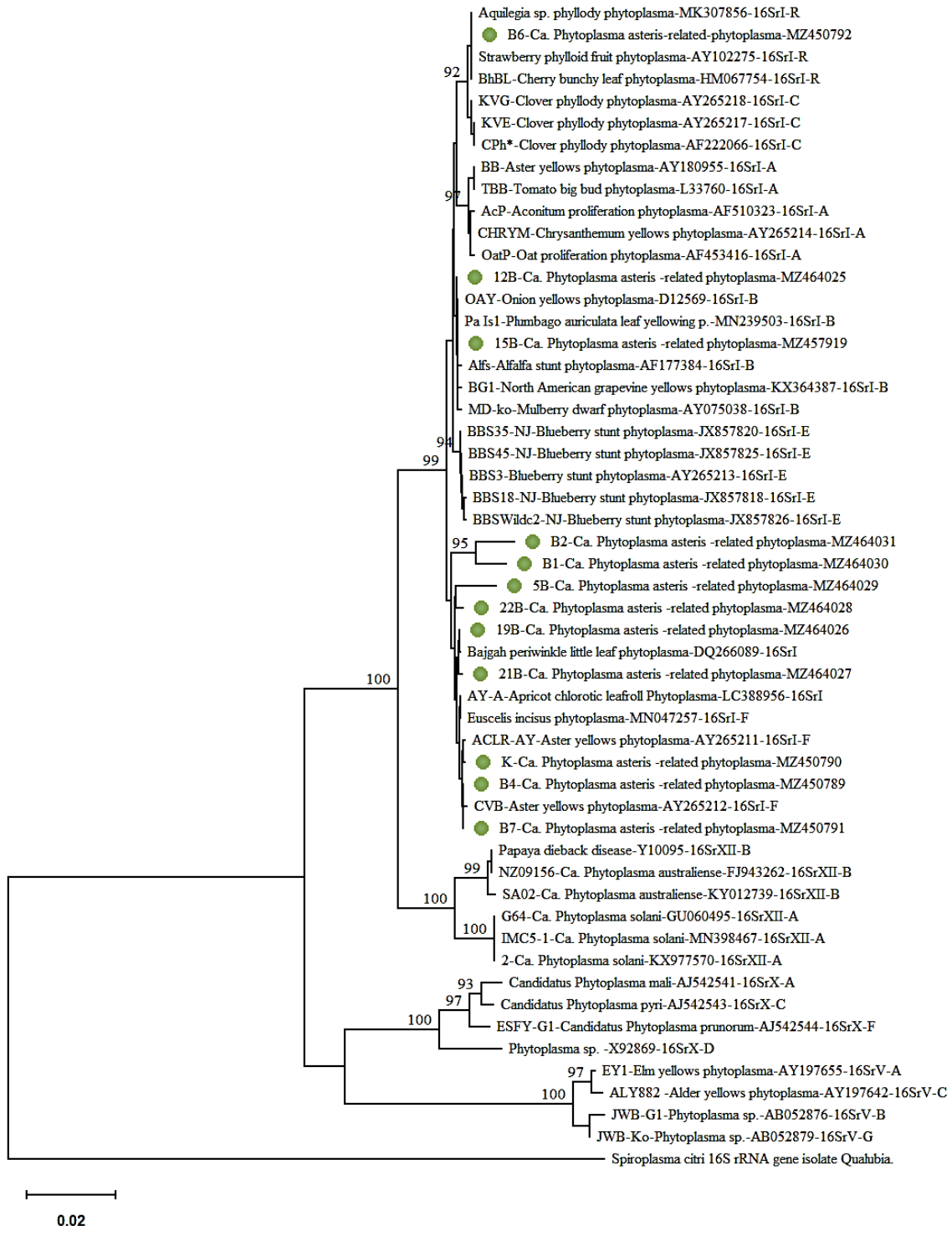

Figure 3. Phylogenetic tree generated using the Neighbor-Joining statistical method, and the substitution model Tamura-3 parameter (T92) of nucleotide sequences of the 16Sr gene of the phytoplasma isolates from insect specimens. A green circle is used to identify phytoplasma isolates in this study. Only values greater than 90% were shown in the bootstrap values on each branch, which was supported by 1000 replicates. *Spiroplasma citri* (accession no AM157769) was used as an outgroup.

In-vitro and *in-silico* PCR-RFLP analysis results

PCR products of 1.2 kb obtained from five individual insects (B4-phy, B6-phy, B7-phy, K-phy and 15B-phy isolates) which are potential phytoplasma vectors, were digested with *TaqI* and *AluI* enzymes *in vitro* conditions, as shown in Figure 4. The profiles were compared with the reference sample ‘’*Ca*. P. asteris’’ related strain cabbage chloranthy (Chlorantie du Chou, in French) CHLL.

Reference aster yellows group and its subgroup sequences were used to compare *in vitro* computer-simulated slaughter profiles of five individual putative insect vectors for which nt sequence analysis was completed. Accordingly, *AluI* and *MseI* endonuclease enzymes were used to separate subgroups in the *i*PhyClassifier (Zhao et al., 2013) software. In the digestion with the *AluI* enzyme, B4-phy, K-phy and B7-phy isolates had identical profiles with the 16SrI-F reference strain and separated from 16SrI-R-16Sr I-B subgroups. B4-phy, K-phy, B7-phy and 15B-phy isolates had the same patterns with each other in the *MseI* enzyme digestion profile. The B6-phy isolate was ideally mirrored to the profile of the 16SrI-R reference isolate and the other phytoplasma subgroups were separated from the 16SrI-B/16SrI-F subgroups (Figure 5).

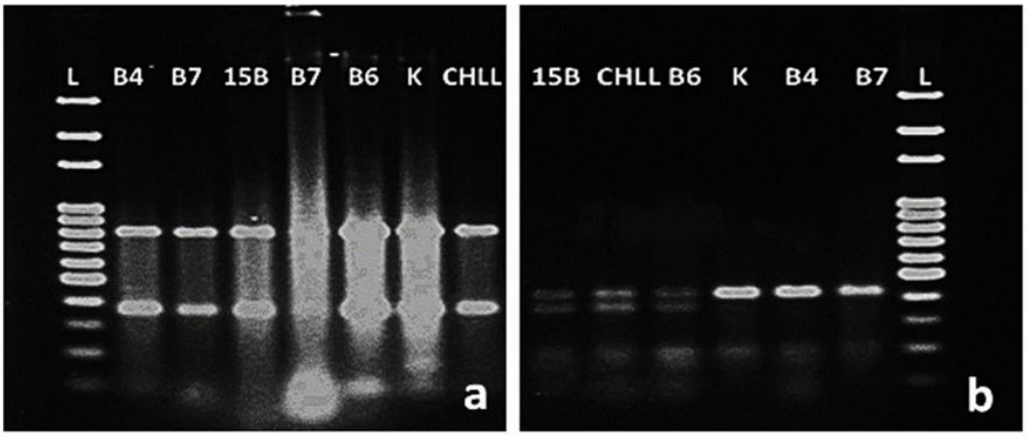

Figure 4. *TaqI* (a) and *AluI* (b) enzyme digestion profiles obtained by amplification of five phytoplasma potential vectors with R16F2n/R16R2 (1.2 kb) primers (B4-phy, *Psammotettix* sp.; B7-phy, *Psammotettix* sp.; K-phy, *Psammotettix* sp.; B6-phy, *P. striatus;* 15B-phy, *C. divaricata*; and reference strain CHLL, 16SrI).

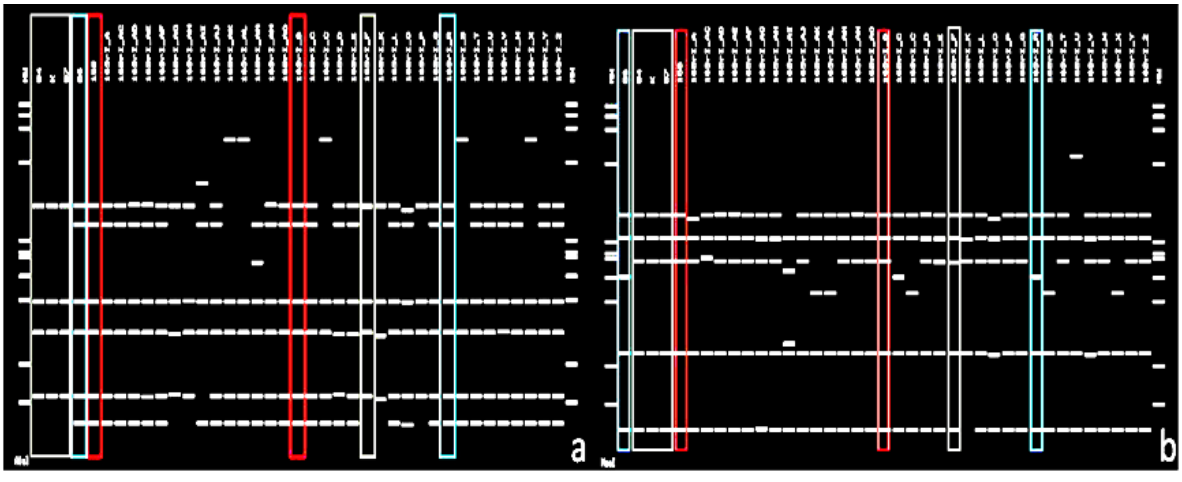

Figure 5. Digest profiles of insect isolates (B6-phy-*P. striatus*, B4-phy-*Psammotettix* sp., K-phy-*Psammotettix* sp., B7-phy-*Psammotettix* sp. and 15B-phy-*C. divaricata*) formed *in silico* with (a) *AluI* and (b) *MseI* enzymes and comparison with other 16SrI aster yellows reference subgroups. Red rectangles indicate the pattern of the 16SrI-B subgroup and the 15B-phy isolate, blue rectangles indicate the pattern of the 16SrI-R subgroup and the B6 isolate, and white rectangles indicate the pattern of the 16SrI-F subgroup with B4-phy, K-phy and B7-phy.

Discussion

Phytoplasmas can infect various economically important crop plants, and they can spread around via numerous hemipteran insect vectors, which provide a great example of tritrophic interactions: host-pathogen-vector (Weintraub & Beanland, 2006; Bertaccini & Lee, 2018). Although chemical pesticides are widely used to control insect vectors and thus prevent the spread of phytoplasmas, total eradication seems to be impossible (Kumari et al., 2019). Also, the lack of studies to determine which insect species can transmit the pathogens limits the design of robust vector control programs. In the present study, we investigated the potential insect vectors and phytoplasmas transmitted by them in carrot production areas in Ankara and Konya, which are the largest carrot-growing provinces in Türkiye.

Although more than 20 leafhopper and planthopper species (Insecta: Hemiptera) have been reported to successfully acquire and transmit AY phytoplasma (strains so far, the aster leafhopper, *Macrosteles quadrilineatus* Forbes, 1885 has been considered primary AY phytoplasma vector (Hoy et al., 1999; Frost et al., 2011). More specifically, in the northeastern USA and Canada, *M. quadrilineatus* and *Scaphytopius irroratus* Van Duzee, 1910 have been determined to be the main vector transmitting the 16SrI-A subgroup phytoplasma and 16SrI-B subgroup phytoplasma, respectively (Lee et al., 2006). Similarly, *M. quadrilineatus* was the most common vector species in carrot production areas of the USA, followed by *Empoasca fabae* (Harris, 1841), *Doratura stylata* (Boheman, 1847), and *Latalus* sp. (Stillson & Szendrei, 2020). Other *Macrosteles* species, *M. quadripunctulatus* (Kirschbaum, 1868) and *M. sexnotatus* (Fallén, 1806) species were found to be potential vectors of the 16SrI-A and 16SrI-B subgroups, while *Macrosteles laevis* (Ribaut, 1927) was identified to be a potential vector of the 16SrXII group in Serbian carrot growing areas (Duduk et al., 2008). Although we determined 13 hemipteran species (mostly belonging to the family Cicadellidae), we did not find any *Macrosteles* spp. in the surveyed areas. However, some *Macrosteles* spp. have been reported among the non-intensive pest populations in the sainfoin cultivation areas of the Ankara and Konya Provinces (Tamer et al., 1997). More specifically, the inability to reach *Macrosteles* spp. populations within the scope of this study may be related to parameters such as the frequency of surveillance, climatic changes, and the diversity of agricultural product patterns. *Psammotettix striatus* was found in both neighboring cities in the present study, however, overall vector fauna even in these two closely located areas seems to be quite different, indicating the importance of local pest control programs to prevent vectors from transmitting phytoplasmas in carrot fields.

Gera et al. (2011) collected a number of leafhopper species such as *Orosius orientalis* (Matsumura, 1914), *Circulifer* sp., *Exitianus capicola* (Stål, 1855), *Neoaliturus fenestratus* (Herrich-Schäffer, 1834) and *Hyalesthes obsoletus* Signoret, 1865 using sticky traps in a carrot field in Israel. However, we did not collect any of these species in the present study. In Serbia, several species belonging to the genera *Psammotettix* and *Anaceratagallia* (especially *P. confinis* and *A. laevis*) have been recorded in phytoplasma-infected carrot fields and the presence of AY (16SrI-A/C) and STOL (16SrXII-A) groups in these genera have been documented (Drobnjaković et al., 2010) whereas *Psammotettix* spp. (including *P. confinis*) sampled in the present study were infected with 16SrI-F/R subgroups. In parallel, *Empoasca* spp. collected from apricot-plum orchards have been reported to contain a 16SrX-B subgroup (Pastore et al. 2004) whereas the phylogenetic tree showed that the phytoplasma was isolated from *Empoasca* sp. from Türkiye carrot fields clustered with 16SrI-F subgroups. These results indicated that a single species or a certain genus can potentially transmit diverse groups of phytoplasmas. In addition, we determined that the 16SrI-B subgroup includes *C. divaricata* and a Psyllidae species, however, none of the collected samples had the 16SrXII subgroup. The 16SrI-F isolate obtained from insects sampled from carrot fields shared a high similarity (99.92%) with the ACLR-AY strain from apricot in Germany (accession: AY265211). This can be explained by the fact that aster yellows have a very wide host range (Kumari et al., 2019).

Although we initially identified *Psammotettix* to genus level based on morphology, molecular identification indicated that the species was *P. confinis* based on BLAST analysis. This clearly shows the usefulness of DNA-based vector identification which also provides early detection of potential vectors in field conditions that have crucial importance. In addition, molecular identification also allows species identification using all developmental stages of insects, contrary to morphological diagnosis which needs adults to make decisions. However, we could not identify the other two species to species level due to the lack of reference sequences, therefore, more and more studies are needed to enlarge the reference sequence database related to vectors of phytoplasma diseases.

Possible vector insects that could be a source of inoculum for phytoplasma infections in carrot-growing areas, as well as the phytoplasma groups transmitted by them, were revealed in this study. More research is needed to identify diseases caused by phytoplasmas in vegetable cultivation areas and characterize their vectors and indirectly control them. Also, an insect cannot be assumed to be a pure vector just because its body contains phytoplasma; therefore, a transmission assay is required in future studies to develop rational control strategies and to provide clear evidence of pathogen transmission in laboratory and field conditions.

Conclusion

The vector-phytoplasma-host complex can explain how they interact with their environments and how they persist, namely, how they fit into ecological niches. Although genetic variation in most phytoplasma subgroups appears to be associated with the ecological isolation of the organisms, genetic diversity in some phytoplasma subgroups (16SrI-A,B) is related to a wide diversity of host plants and insect vectors (McCoy et al., 1989). The families Aphrophoridae, Cicadellidae, Psyllidae and Delphacidae were identified as potential phytoplasma insect vectors in this study, which were sampled from carrot-growing areas. However, only members of the Cicadellidae and Psyllidae have been found to carry the phytoplasma of the aster yellows group in their natural habitat. In these regions where the continental climate is dominating, there is a greater requirement for the identification of vector insects that are effective in the transmission of phytoplasma diseases. On the other hand, identifying the phytoplasma groups and subgroup populations that can adapt to these environments and dominate their ecological niches may reveal more possibilities for developing an agricultural control strategy against these phytopathogens.

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