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Molecular Detection of Grape Decline Phytoplasma in Leafhopper Species Associated with Infected Grapevines in Iran

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Following recent detection of '*Candidatus* Phytoplasma fraxini', '*Ca.* P. aurantifolia', '*Ca.* P. solani' and '*Ca.* P. phoenicium'-related strains in Iranian vineyards, a survey was conducted in 2016–2017 in vineyards located in Qazvin province of Iran. Nested PCR analysis was performed to identify phytoplasma strains in leafhopper species. The overall, less than 5% of grapevine trees shows phytoplasma symptoms and phytoplasma was detected in all symptomatic samples. Phytoplasma DNA was detected in two leafhopper species. Most leafhopper species in which phytoplasma DNA were detected are recorded to be grass feeders, among which, Stolbur phytoplasma vectors, i.e. *Psammotettix alienus* (Dahlbom 1851) and *Agallia ribauti* (Ossiannilsson, 1938), showed a strong potential ability for '*Ca.* P. solani' transmission in Qazvin vineyards. The possibility of phytoplasma transmission between leafhoppers and grapevines is discussed.

Keywords: Phytoplasma, leafhoppers, grapevine, decline, transmission.

The presence of phytoplasmas associated with yellows disease in *Vitis vinifera* L. in Iran was reported based on symptoms, nested PCR/RFLP detection or sequencing of 16Sr RNA gene. The phytoplasmas, identified so far, are '*Candidatus* Phytoplasma solani'-related strains and more recently a strain enclosed in 16SrIX-C subgroup (Ghayeb Zamharir et al., 2017). Four different phytoplasmas ('*Candidatus* Phytoplasma fraxini', '*Ca.* P. aurantifolia', '*Ca.* P. solani' and '*Ca.* P. phoenicium) have been detected in Iranian vineyards that had grape decline symptoms. Among these phytoplasmas, '*Ca.* P. solani'-related strains were the most prevalent (Ghayeb Zamharir et al., 2017).

Phytoplasmas previously called mycoplasma-like organisms, are wall-less prokaryotes belonging to the class of mollicutes (McCoy et al., 1989; Lee et al., 2010). Phytoplasmas are transmitted by grafting, dodders and phloem-feeding insects, most of which are Auchenorrhyncha, such as leafhoppers (Schvester et al., 1961; Nault, 1990; Fernando et al., 1993; Olivier et al., 2012). The spread and progress of grape decline are influenced

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by inoculum density and activity of vectors. Therefore, information on potential vectors and their population dynamics, along with data on inoculum sources are needed for risk assessment and progress prediction of grape decline, which finally will end up in successful control methods (Weber and Maixner, 1998).

Detection of phytoplasma in insect vectors by molecular methods is a step toward identification of a vector, however, this does not provide a strong evidence of its vectoring ability, as many insects can acquire phytoplasmas and be considered as 'carriers' (Hogenhout et al., 2008). Meanwhile, some insect species can be competent 'vectors', i.e., can acquire phytoplasmas from a plant and successfully transmit them to another plant (Weintraub and Jones, 2009). Vectoring ability is the result of a successful specific tritrophic interaction between the phytoplasma, the insect vector and the host plant (Weintraub and Beanland, 2006; Hogenhout et al., 2008). Therefore, biological transmission assays are, the only way to ascertain phytoplasma transmissibility. These assays are time-consuming and require a big colony of phytoplasma-free insects and also phytoplasma-susceptible plants on which the insects shall feed and survive (Daire et al., 1992; Maixner et al., 1995; Sforza et al., 1998). Therefore, they are not practical for large-scale epidemiological studies (Gatineau et al., 2001).

Essential step to study phytoplasma disease in vineyards is to determine the biodiversity of leafhoppers associated with vineyards, and identify the phytoplasma strains associated with their potential insect vectors. The main objectives of this study were: 1) to detect phytoplasmas in leafhopper species that were collected from Iranian vineyards infected with grape decline, and 2) to identify phytoplasma strains present in these leafhoppers.

Materials and Methods

Insect samples

During 2016 and 2017, vineyards of Qazvin province (Iran) were visited at least once a month to select vineyards showing characteristic symptoms (e.g., yellowing and leaf scorch). Six commercial vineyards were selected in which yellow sticky traps (20×10 cm) (Russel Company, UK) were installed on vines branches at a height of 75 cm from ground. In each vineyard, five independent rows (one cultivar/row), with symptomatic grapevines were selected (Table 1).

Installation of sticky traps started on May. Sticky traps were collected fortnightly and new traps were replaced until September. Each collected trap was transferred to laboratory in a separate plastic bag, so the traps would not stick to each other. Traps were checked under stereomicroscope, and the leafhoppers were detached along with surrounding sticky card using a sharpe scalpel. The samples were transferred into xylene (dimethylbenzene) to dissolve the glue and release the leafhopper. The leafhoppers were kept in ethanol until identification, followed by molecular studies.

Leafhopper identification

Leafhopper species were identified according to the habitus characters and male genitalia, using original descriptions and published illustrations (Emeljanov, 1967; Dlabola, 1974; Biedermann and Niedringhaus, 2009).

Table 1

The sources of each leafhopper population and the quantity of the insects belonging to each population

Population no.	Geographical coordinates of studied gardens	Quantity of the insects	Year	Insect spices
1	39S 381746 3986603	10	2016	*P. shirazicus*
2	39S 381746 3986603	15	2016	*A. ribauti*
3	39S 381746 3986603	12	2016	*P. alienus*
4	39S 381558 3986737	17	2016	*P. alienus*
5	39S 381558 3986737	14	2016	*A. ribauti*
6	39S 381558 3986737	10	2016	*P. shirazicus*
7	39S 381558 3986737	15	2016	*P. shirazicus*
8	39S 380665 3986734	14	2016	*P. alienus*
9	39S 380665 3986734	15	2016	*P. shirazicus*
10	39S 382283 3982474	17	2016	*A. ribauti*
11	39S 382283 3982474	10	2016	*A. ribauti*
12	39S 382283 3982474	14	2016	*P. shirazicus*
13	39S 381793 3982038	17	2016	*P. alienus*
14	39S 381793 3982038	22	2016	*A. ribauti*
15	39S 381793 3982038	15	2016	*P. alienus*
16	39S 381746 3986603	14	2017	*P. shirazicus*
17	39S 381746 3986603	12	2017	*A. ribauti*
18	39S 381558 3986737	14	2017	*A. ribauti*
19	39S 381558 3986737	15	2017	*A. ribauti*
20	39S 382283 3982474	17	2017	*P. alienus*
21	39S 380665 3986734	20	2017	*A. ribauti*
22	39S 382283 3982474	18	2017	*P. alienus*
23	39S 382283 3982474	17	2017	*P. shirazicus*
24	39S 381793 3982038	15	2017	*P. shirazicus*
25	39S 381793 3982038	17	2017	*P. alienus*

DNA isolation

DNA was extracted from insects according to Maixner et al. (1995). Insects tissue was ground in extraction buffer (100 mMTrisHCl at pH 8.0, 2% cetyltrimethylammonium bromide (CTAB), 1.4 M NaCl, 20 mM EDTA, and 0.2% 2-mercaptoethanol) at a ratio of 1:5 (wt/vol, tissue/buffer). The slurry was incubated for 20 min at 60 °C and centrifuged for 10 min at 3,000 × *g*. The supernatant was collected and extracted with an equal volume of chloroform/isoamyl alcohol (24:1, vol/vol), followed by centrifugation and precipitation with 1 volume of isopropanol. Following 30-min incubation at –20 °C, the DNA was pelleted at 12,000 × *g* for 30 min. The pellet was washed twice with 70% ethanol and resuspended in 50 μl of 10 mMTris and 1 mM EDTA at pH 8.0 (TE).

Nested PCR

DNA extracts were diluted 1:10 with 10 mM Tris-Cl, pH 8.5, and used as a template in PCR to amplify the 16S rRNA-encoding gene F2nR2 fragment with primers R16F2n/R16R2. In this order, PCR amplification with universal phytoplasma primers P1/Tint (Deng and Hiruki, 1991; Smart et al., 1996) followed by R16F2n/R2, R16mF1/mR1 (Gundersen and Lee, 1996), R16(I)F1/R1 (Lee et al., 1994) or 6R758f/16R1232r (= M1/M2) primers (Gibb et al., 1995) in nested polymerase chain reaction (PCR) assays using as template the PCR products was diluted with sterile distilled water (1: 30).

DNA sequencing and phylogenetic tree

Selected R16F2n/R2 (1,248 bp) and M1/M2 amplified fragments from phytoplasma detected in grapevine leafhoppers were directly sequenced. Sequences were trimmed and compared with sequences of representative phytoplasma strains detected in grapevine with decline symptoms and other 16Sr phytoplasma groups (Wei et al., 2008) recorded in GenBank, using the BLAST Program (http://www.ncbi.nlm.nih.gov) and grouped into phylogenetic tree using the Mega7 Program (Tamura et al., 2011). Sequence alignments were performed, using Clustal X (Thompson et al., 1997). The sequences obtained from grapevine samples were then trimmed to the shortest one and employed in phylogenetic analyses using all the reported '*Candidatus* Phytoplasma' species officially described using *Acholeplasma laidlawii* as the out group. The analysis was replicated 1,000 times to estimate stability and support for the inferred clades. The evolutionary history was inferred using the Neighbor-Joining method. The evolutionary distances were computed using the Maximum Composite Likelihood method and evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

Restriction fragment length polymorphism (RFLP) analyses

For RFLP analysis 3 µl (about 300 ng of DNA) of R16F2n/R2 or M1/M2 PCR products of each positive nested PCR product was separately digested with enzymes (*Tru1*I, *Taq*I, RsaI and *Tsp509*I) at 65 ºC, as recommended by the manufacturers (Fermentas, Vilnius, Lithuania and New England Biolabs, respectively). Restricted fragments

were separated through 3% Agaros gel electrophoresis in TAE buffer. Patterns obtained were recorded in a Kodak gel documentation unit.

Results

Leafhopper identification

Three different leafhopper species were identified, as follows: *Platymetopius shirazicus* Dlabola 1974 (Hom.: Cicadellidae) (Fig. 1, Table 1), *Agallia ribauti* Ossiannilsson 1938 (Hom.: Cicadellidae) (Fig. 1) and *Psammotettix alienus* Dahlbom 1851 (Hom.: Cicadellidae) (Fig. 1). The species *A. ribauti* and *P. alienus* can transmit Stolbur phyroplasma.

Detection and identification of phytoplasmas

Among more than 25 leafhopper populations collected from Qazvin vineyards during 2016 and 2017, three species of three different genera were identified and submitted to PCR analysis. Phytoplasma DNA was detected in seven samples belonging to *A. ribauti* and *P. alienus* species.

RFLP analysis of the R16F2n/R2 and M1/M2 products with *Taq*I and *Mse*I (Fig. 2) indicated that the phytoplasmas from grapevine leafhoppers in Qazvin province (Iran) are mixed infection of two different group 16SrII (‘*Candidatus* Phytoplasma aurantifolia’) and 16SrXII group (*Ca.* P. solani’, “stolbur”) (Fig. 2). But single infection was observed in three populations of seven positive populations that related to *A. ribauti* species. Sequencing and blast analysis of these samples indicated that this phytoplasma related to the 16SrII (‘*Candidatus* Phytoplasma aurantifolia’) group and shared 100% similarity

Fig. 1. *Platymetopius shirazicus* Dlabola, 1974,
Agallia ribauti Ossiannilsson, 1938, Psammotettix *alienus* (Dahlbom, 1850)

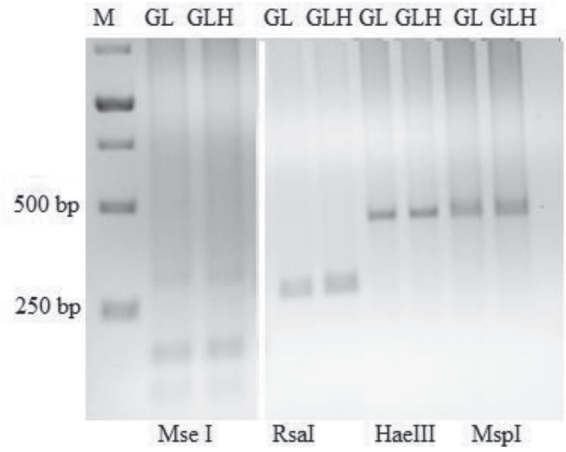

Fig. 2. M1/M2 RFLP analysis of phytoplasma collected from grape leaf (GL) and leafhoppers (GLH) in vineyards

with phytoplasma isolate that have been collected from grape vine decline in this area (accession numbers MH342005 and MH342006). The sequence of phytoplasma isolates that amplifies from grapevine leafhoppers in Qazvin province deposited in gene bank (NCBI) as MH342002, MH342003 and MH342004 accession numbers. Phylogenetic analysis also clustered grapevine leaf and leafhoppers phytoplasma strains with 16SrII phytoplasma group (Fig. 3).

Discussion

Phytoplasmas are important insect transmitted pathogens attack more than hundreds of commercial and native plants, causing minor to extensive damage (Kirkpatrick, 1992; Davies et al., 1992; Lee et al., 1996, 2010). Insect vectors of phytoplasma diseases including primarily leafhoppers, planthoppers, and psyllids, have been identified for relatively few phytoplasma diseases (Kirkpatrick, 1992; Davies et al., 1992; Maixner, 2010).

Surveys of insects found on and around affected plants and focusing on the phloem-feeding Hemiptera taxa are the first step towards determining vectors of a given phytoplasma disease (Davies et al., 1992). In this order in this study we have collected leafhoppers in Iranian vineyard that have been infected by phytoplasma disease.

Grape decline and yellows phytoplasma disease is one of important phytoplasma disease in all over the world (Sforza et al., 1998; Rott et al., 2007; Quaglino et al., 2013) that is an emerging threat to vineyards in Iran. The extensive survey on phytoplasma detection and identification in Iran vineyards indicated the presence of '*Candidatus* Phytoplasma fraxini', '*Ca.* P. phoenicium', '*Ca.* P solani' and '*Ca.* P. aurantifolia'– related strains (Ghayeb Zamharir et al., 2017). The presence of these phytoplasmas is probably related to the presence of those in the Iranian plants and trees, however the work about grapevine phytoplasma insect vectors of all the phytoplasmas detected in Iran vineyards is not studied yet (Ghayeb Zamharir et al., 2017).

Three leafhopper species including *P. shirazicus*, *A. ribauti* and *P. alienus* were collected from Qazvin province (Iran). *P. shirazicus* was recorded as a pest on almond with mild economic importance by Rajabi (1991). The species is known as an endemic species to Iran, distributed in central Alborz mountain and southwest of the country (Mozaffarian and Wilson, 2016). Due to the lack of any report on the serious damage of the species, it was suggested by Mozaffarian (2018) to evaluate the real pest status of the

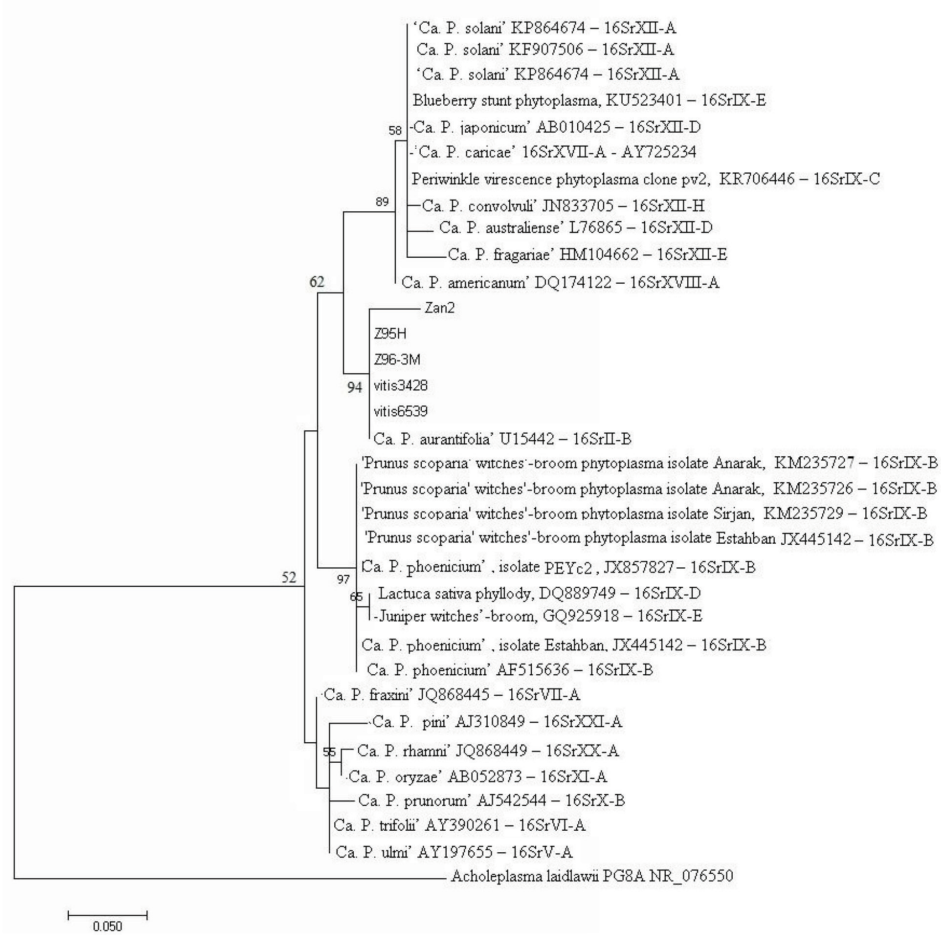

Fig. 3. Molecular Phylogenetic analysis by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood (-476.95) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 34 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 126 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Tamura et al., 2004)

species more thoroughly. Among the collected leafhoppers, only *A. ribauti* and *P. alienus* species were PCR assay positive. *A. ribauti* was already recorded by Kheyri (1989) as a pest with minor economic importance on the leaves of sugar-beet in Iran. In other parts of the world, *A. ribauti* was shown to be able to transmit stulber phytoplasma to *Vicia faba* seedlings and also positive to those phytoplasmas on carrot (Drobnjaković et al., 2010). Kheyri (1989) mentioned *P. alienus* as a pest with little economic importance on leaves of sugar-beet. The species is known as s phloem-feeding vector in vineyards of Italy (Minuz et al., 2013). Drobnjaković et al. (2010) detected stolbur phytoplasmas in the specimens of this species. Landi et al. (2013) showed the inoculation of the species to phytoplasma 16SrI-B subgroup. Result of RFLP analysis shows that four out of seven leafhopper population collected from Qazvin vineyards had mixed infection of "stolbur" with 16SrII ('*Candidatus* Phytoplasma aurantifolia'). In order to lack of sequencing data for stolbur phytoplasma associated with vineyards leafhoppers, its impossible to confirm that association phytoplasma is causal agent of grape yellows and decline or not. But sequencing and blast analysis of phytoplasmas that were detected in *A. ribauti* species indicated that a phytoplasma related to the 16SrII ('*Candidatus* Phytoplasma aurantifolia') group (accession numbers MH342005 and MH342006) shared 100% similarity with grape decline phytoplasma isolates (accession numbers MH342002, MH342003).

Recently studies in the field of biology, ecology, vector relationships, and epidemiology of phytoplasma diseases has gone into understanding individual phytoplasma diseases and their vectors. But many questions about phytoplasma plant disease-vector relationships is unsolved yet (Lee et al., 1996, 2010; Maixner, 2010) that could limit the capacity of managers to make informed decisions to protect crops and endangered indigenous plants. These results demonstrate the putative vectors of grape decline and yellows phytoplasma disease that can help to perform the transmission tests and understand grape phytoplasma pathosystems.

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