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Detection and molecular identification of '*Candidatus* phytoplasma trifoli' infecting some cultivated crops and vegetables in West Azarbaijan province, Iran

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Abstract Phytoplasma-like symptoms were observed in plants of pepper (*Capsicum annum*), red cabbage (*Brassica oleracea)*, common bean (*Phaseolus vulgaris)*, maize (*Zea mays*), alfalfa (*Medicago sativa*), cucumber (*Cucumis sativus*), sunflower (*Helianthus annus*), tomato (*Solanum lycopersicum*) and squash (*Cucurbita* spp.) in West Azarbaijan province of Iran. The major symptoms observed were little leaf, witches' broom, yellowing, phyllody, big bud and dwarfing of plants. DNA fragments of approximately 1800 and 1250 bp were amplified from 36 samples using universal primer pairs P1/P7 and R16F2n/R16R2 in first round and nested PCR, respectively. The phytoplasmas detected in maize, cucumber and tomato were characterized and differentiated through sequence analysis of PCR-amplified rDNA. These phytoplasmas were identified as members of the clover proliferation phytoplasma group (16SrVI group) and classified in subgroup 16SrVI-A. To the authors' knowledge, this is the first report of the occurrence of 16SrVI phytoplasmas in maize.

Keywords Clover proliferation · Nested PCR · Maize · Tomato · Cucumber

Phytoplasmas were first discovered in 1967 and were named mycoplasma-like organisms or MLOs (Doi et al. 1967). They are non-culturable prokaryotic microorganisms that cause several hundred diseases of various plants (McCoy et al. 1989; Firrao et al. 2005) and are transmitted by phloem-feeding leafhoppers and planthoppers (Christensen et al. 2005; Weintraub and Beanland 2006; Bertaccini et al. 2014). Plants infected by phytoplasmas exhibit a variety of symptoms that suggest profound disturbances to the normal plant physiology. Phytoplasma diseases are increasingly important worldwide, with a high economic impact on crop production and quality, costing millions of dollars to control (Agrios 2005; Bertaccini 2007). Because of the high economic losses, in particular those of cultivated crops, annuals and high-value vegetables, detection of phytoplasmas is very important. Hence this study was planned to identify the phytoplasmas associated with some cultivated crops and vegetables in West Azarbaijan province of Iran, because little is known about phytoplasma diseases of plants in West Azarbaijan province.

Cultivated crops and vegetable samples showing typical symptoms of phytoplasma infection were collected during the 2013 and 2014 growing seasons (Table 1) from the cities of Urmia, Khoy, Salmas and Piranshahr in West Azarbaijan province. Leaf petioles and midribs from healthy controls (grown from seeds in insect proof glasshouses) and from naturally-infected hosts were used for DNA extraction. Approximately 1.5 g of fresh tissue was used for each extraction, according to the method described by Zhang et al. (1998). The universal primer pair P1/P7 (Schneider et al. 1995) was employed in first round PCR to amplify an 1800 bp fragment of 16S rDNA. A 30-fold dilution of the first round PCR product was then used as template for nested PCR using primer pair R16F2n/R16R2 which amplified an internal fragment of 1250 bp from the 16S rDNA (Lee et al. 1993). The total volume of 20 μl PCR reaction mixtures contained 20 ng DNA, 0.2 mM of each dNTP (Cinnagen, Iran), 1.6 mM $MgCl_2$, 1U of *Taq* DNA polymerase (Cinnagen, Iran), 0.5 μl of each primer pair (20 pmol/μl) and 1X polymerase buffer.

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Table 1 Cultivated crops and vegetables showing phytoplasma like symptoms

Common name	Scientific name	Plant family	Location of sampling	Number of collected samples	Symptoms	Number of positive samples
Pepper	*Capsicum frutesens* L.	Solanaceae	Urmia-Laladio-torab	1	dwarfing	1
			Urmia-Garajalar	1	Witches' broom	1
			Urmia-Tazeh kand	1	Little leaf	0
			Khoy-Zaviyeh hasan khan	1	Little leaf	0
Red cabbage	*Brassica oleracea* L.	Brassicaceae	Urmia-Topragh Ghala	1	Little leaf	0
Common bean	*Phaseolus vulgaris* L.	Fabaceae	Urmia-Nazloo	1	Little leaf	1
			Urmia-Targavar	1	Little leaf	0
Maize	*Zea mays* L.	Gramineae	Urmia-Chenaghloo	1	Yellowing and dwarfing	1
			Urmia-Nazloo	1	Yellowing and dwarfing	1
			Khoy-Zaviyeh hasan khan	1	Yellowing and dwarfing	0
Alfalfa	*Medicago sativa* L.	Fabaceae	Urmia-Chenaghloo	1	Little leaf and dwarfing	0
			Khoy-Zaviyeh hasan khan	1	Little leaf and dwarfing	0
Cucumber	*Cucumis sativus* L.	Cucurbitaceae	Urmia-Keshtiban	2	Phyllody	2
			Urmia-Tazeh kand	1	Phyllody	1
Sunflower	*Helianthus annuus* L.	Asteraceae	Salmas-Minas	1	Little leaf and witches' broom	0
			Khoy-Zaviyeh hasan khan	1	Little leaf and witches' broom	0
Tomato	*Solanum lycopersicum* L.	Solanaceae	Urmia- Keshtiban	4	Big bud, little leaf and purple leaves	3
			Urmia-Chachal ali	2	Purple leaves	2
			Urmia-Abajaloo	1	Big bud	1
			Urmia-Tazeh kand	2	Big bud, sterility of flowering	1
			Urmia-Jahatloo	1	Big bud	1
			Urmia-Heydar beigloo	1	Big bud	1
			Urmia-Sheikh sarmast	1	Big bud	1
			Urmia-talatappeh	1	Big bud, sterility of flowering	1
			Urmia-Igdir	1	Purple leaves, fruit malformation	1
			Piranshahr	1	Big bud	0
Squash	*Cucurbita* spp.	Cucurbitaceae	Urmia-Tazeh kand gheshlagh	2	Phyllody	2
			Urmia-Tazeh kand	1	Phyllody	1
			Urmia-Nazloo	1	Phyllody	1

The reaction mixtures were subjected to 35 cycles at the following conditions: First round PCR (35 cycles): 1 min (3 min for the first cycle) for denaturation step at 94 °C, 1 min for annealing at 57 °C and 1.5 min (10 min for the last cycle) for primer extension step at 72 °C. Second round nested PCR (35 cycles): 2 min (5 min for the first cycle) for denaturation step at 94 °C, 1 min for annealing at 57 °C and 2 min (10 min for the last cycle) for primer extension step at 72 °C. The PCR products were analyzed by electrophoresis in a 1 % agarose gel using Tris-Borate EDTA (TBE) buffer, and stained with 5 μg/ml ethidium bromide. An ultraviolet (UV) transilluminator was used to visualize PCR products (Smart et al. 1996). PCR products from nested PCR were sent for sequencing directly. Sequencing was performed by Macrogen (South Korea) on both strands. A phylogenetic tree was constructed using the neighbor-joining (NJ) method, with MEGA5 software (Tamura et al. 2011) and subjected to bootstrap analysis (using 1000 replicates).

Thirty-six samples of cultivated crops and vegetables with phytoplasma-like symptoms were collected from different parts of West Azarbaijan province. The symptoms varied with the host plant and the most characteristic symptoms were witches' broom, dwarfing, little leaf, big bud, phyllody, purple leaves and yellowing. However leaf malformation and sterility of flowers were also detected in some samples (the symptoms are summarized in Table 1). Pepper, maize, cucumber, tomato, common bean and squash were found to be positive by nested PCR, whilst red cabbage, alfalfa and sunflower and healthy plants were negative. The 1250 bp amplicons obtained were sequenced and the nucleotide sequences of the phytoplasmas

detected in three plants (tomato, cucumber and maize) were deposited in GenBank (with accession numbers of KT807466, KT807467, KT807468, respectively). BLAST searches showed the highest homology (99 %) of the 16S rDNA sequences of the three detected phytoplasmas to members of the 16SrVI group (‘*Candidatus* phytoplasma trifolii’). Phylogenetic analysis of sequences presented in this survey together with 14 related phytoplasmas and six representative strains in 16SrVI subgroups (A,B,C,D,E,H) from GenBank clustered the phytoplasmas with 16SrVI group phytoplasmas (Fig. 1).

Phytoplasmas have previously been identified in maize, including *‘Ca*. phytoplasma solani’ (16SrXII group) in Serbia (Duduk and Bertaccini 2006) and Bulgaria (Genov et al. 2014), associated with corn reddening and a 16SrI phytoplasma *‘Ca*. phytoplasma asteris’ associated with maize bushy stunt (Arocha and Jones 2010) in Serbia. In this study, a 16SrVI group phytoplasma was found in maize showing yellowing and stunting. To our knowledge this is the first report of a *‘Ca*. Phytoplasma trifolii’ isolate affecting maize. In cucumber, Salehi et al. (2006) and Asghari Tazehkand et al. (2009) previously found a phytoplasma of 16SrII group associated with phyllody in central provinces and West Azarbaijan province of Iran. Asghari Tazehkand et al. (2010) also reported a phytoplasma of 16SrVI group from Kerman province of Iran. Dehghan et al. (2014) and Esmailzadeh Hosseini et al. (2015) also found a phytoplasma of 16SrVI group associated with phyllody in greenhouse cucumbers in Fars and Yazd provinces of Iran. In this study we found a 16SrVI group phytoplasma in cucumber in West Azarbaijan province. In tomato, Lee et al. (2000) reported 16SrI (SrI-A, SrI-B), 16SrII (SrII-E), and 16SrVI (SrVI-A) phytoplasmas associated with big bud symptoms. Ghandi et al. (2003), Rashidi et al. (2005) and Du et al. (2013) found 16SrVI group phytoplasmas in Jordan, Isfahan province of Iran and China, respectively on tomato showing big bud symptoms. Xu et al. (2013) found a 16SrII group phytoplasma on tomato in China. In this study we found ‘*Ca*. Phytoplasma trifolii’ in tomatoes showing sterility of flowering, little leaf, purple leaf and big bud symptoms for the first time from West Azarbaijan province of Iran. No phytoplasma was detected in red cabbage, pepper, alfalfa and sunflower plants suggesting that the phytoplasma-like symptoms in these plants can be caused by other factors such as physiological disorders.

Phytoplasma-associated diseases have spread worldwide, and in several cases are associated with severe epidemics of quarantine importance. Many cultivated plants are affected by phytoplasma infections not only in countries where agriculture is still not very well advanced, but also in the so called more advanced countries where these pathogens are severely damaging both herbaceous and woody plants (Bertaccini and Duduk 2009). Phytoplasmas are considered to be amongst the most important plant pathogens reducing the productivity of several economic crops and their detection is very important.

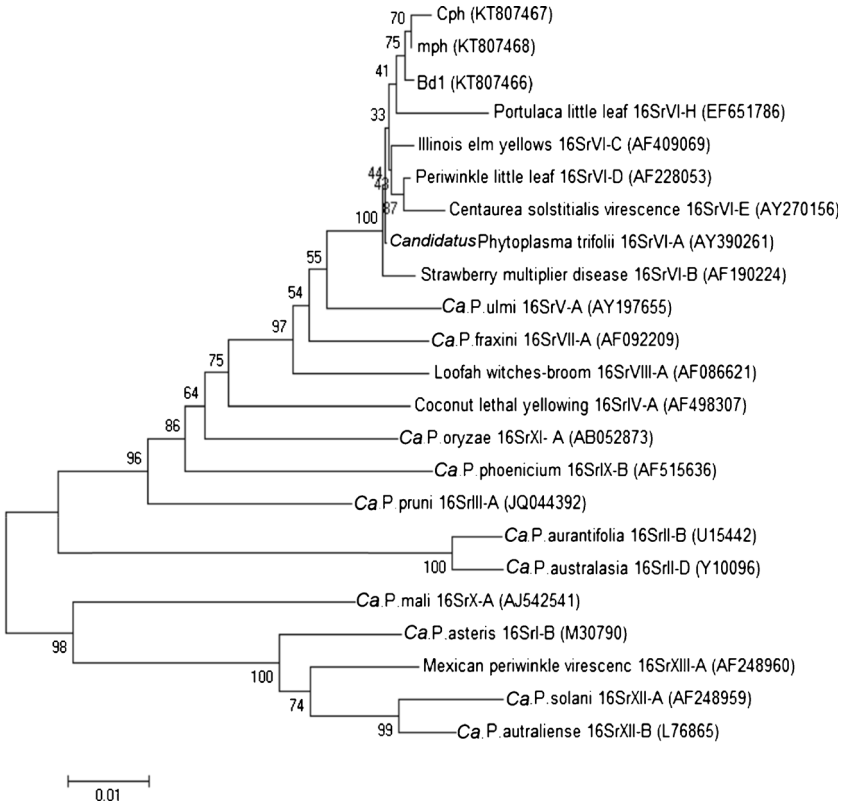

Fig. 1 Phylogenetic tree constructed by the neighbor joining method of 16S rRNA gene sequences from 20 phytoplasma sequences obtained from GenBank and phytoplasmas identified from tomato (Bd1), Cucumber (Cph), and corn (mph). *Numbers* at the nodes are bootstrap values based on 1000 repetitions. GenBank accession numbers for sequences are given in parentheses

Results of this study can facilitate further work on ecology, epidemiology and diversity of phytoplasmas in cultivated crops and vegetables in Iran. To the authors' knowledge, this is the first report on the occurrence of a phytoplasma disease on maize in West Azarbaijan province and in Iran, and the first report of a 16SrVI subgroup phytoplasma occurrence on maize worldwide.

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