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Detection and Molecular Characterization of '*Candidatus* Phytoplasma Trifolii', a Member of the Clover Proliferation Grup, Infecting Tomato Plants from Iğdır Province in Turkey

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ABSTRACT

The tomato plant exhibiting leaf rolling, witches' broom, distorted and elongated flower's sepals in Iğdır province, Turkey, was observed. Total DNA extraction was performed from the symtomatic fresh tomato sample. All DNAs were subjected to Direct and Nested polymerase chain reaction (PCR) with universal primer sets that amplified the 16S rRNA of phytoplasmas. PCR products were purified from agarose gel and cloned into the pGEM T-Easy cloning vector. Recombinant plasmids were introduced into the prokaryotic cloning bacteria by electroporation. Plasmid isolation was performed by selecting one of the positive clones randomly and sequencing was performed by Next Generation Sequencing (NGS). Sequencing results revealed that the 16S rRNA gene associated with phytoplasma was 1251 nucleotides in size, and this sequence was denominated as 'Iğdır 10' isolates and recorded in the GenBank under the MT344968 accession number. The virtual restriction fragment length polymorphism (V-RFLP) and phylogenetic analysis of the 16S rDNA sequence confirmed that the cause of disease in infected tomato plants was '*Candidatus* Phytoplasma trifolii' ('*Ca*. P. trifolii') (16SrVI-A, Clover proliferation group), with a 1.00 similarity coefficient. This present study is the first report of '*Ca*. P. trifolii' and its nucleotide sequence analysis in naturally infected tomato in Iğdır province.

Türk Tarım – Gıda Bilim ve Teknoloji Dergisi, 8(12): 2533-2540, 2020

Türkiye'de Iğdır İli Domates Bitkilerini Enfekte Eden Clover Proliferation Grup Üyesi Candidatus Phytoplasma Trifolii'nin Tespiti ve Moleküler Karakterizasyonu

MAKALE BİLGİSİ

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ÖZ

Iğdır ilinde (Türkiye) şekli bozulmuş ve uzamış çiçek taç yaprakları, yaprak kıvrılması ve cadı süpürgesi simptomları sergileyen domates bitkisi görülmüştür. Belirti gösteren taze dometes örneklerinden toplam DNA extraksiyonu yapılmıştır. Tüm DNA'lar fitoplazmaların 16S rRNA'sını amplifiye eden spesifik üniversal primer setleri ile Direct ve Nested polimeraz zincir reaksiyonuna tabi tutulmuştur. PCR ürünleri agaroz jelden saflaştırılmış ve pGEM T-Easy klonlama vektörüne klonlanmıştır. Rekombinat plazmidler, prokaryotik klonlama bakterisine elektroporasyon ile aktarılmıştır. Pozitif klonlardan rastgele birisi seçilerek plazmid izolasyonu ve akabinde yeni nesil dizileme (NGS) ile dizileme gerçekleştirilmiştir. Dizileme sonuçları, fitoplazma ile ilişkili 16S rRNA geninin 1251 nükleotit uzunluğunda olduğunu ortaya çıkarmış ve bu dizi 'Iğdır 10' olarak isimlendirilerek MT344968 ulaşım numarası ile gen bankasına kaydedilmiştir. 16S rDNA dizisinin virtual restriction fragment length polymorphism (V-RFLP) ve filogenetik analizleri, infekteli domates bitkilerinde hastalık nedeni ajanın 1.00 benzerlik katsayısı ile '*Candidatus* Phytoplasma trifolii' (16SrVI-A, Clover proliferation grup) olduğunu doğrulamıştır. Mevcut bu çalışma, Iğdır ilinde doğal olarak enfektelenmiş domates bitkisinde "*Ca*. P. trifolii" nin ve onun nükleotit dizi analizinin ilk raporudur.

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Introduction

Tomato, originated from South America, is one of the most produced, consumed, and traded agricultural crops and is an indispensable part of the human diet. Tomato is a valuable crop worldwide. Besides the fresh consumption of tomatoes, its use as a raw material in the food industry increases its importance (Keskin and Gül, 2004; Demiray and Tülek, 2008). Turkey ranks fourth in world tomato production following China, India, and the USA (FAO, 2016). Based on the TUIK data, 64.76% of 12,6 million tons of tomatoes production in Turkey are produced as table tomatoes. In Iğdır province, 46.5 thousand tons of table tomatoes are produced from 15.3 thousand decares (TUIK, 2016). The Iğdır province has a 1.22% share of the total table tomato production areas, equivalent to 0.57% of the table tomato production (Karadaş and Ertürk, 2016).

Production of tomato is limited due to its exposure to most pathogens including phytoplasma. Phytoplasmas are highly dangerous pathogens from an ecological and economic standpoint. Phytoplasmas are members of the genus '*Candidatus*' of the Acholeplasmataceae family in the Mollicutes class. It possesses destructive effects at more than 700 plant species in nature worldwide including many cultivated plants, ornamental plants, fruit trees, and various timber and shade trees (Bertaccini et al., 2014; Dermastia et al., 2017). Extensive classification of phytoplasmas is accomplished by RFLP analysis of the amplified 16S rRNA gene by the PCR assays. If the 16S rRNA gene sequence (>1200 bp) of '*Ca*. phytoplasma' species show less than 97.5% similarity to the previously identified gene sequences, a new '*Ca*. phytoplasma' species is proposed. According to this approach, phytoplasmas are divided into 33 groups and more than 40 subgroups (IRPCM, 2004; Arocha et al., 2005).

Phytoplasmas are firmly aphid-mediated transmission pathogens in persistent mode, especially with leafhoppers, planthoppers, and psyllids fed with stylets from phloem tubes (Suzuki et al., 2006) as well as parasite plants (Shimizu and Aoki, 2019). Phytoplasmas are Gram-positive prokaryotes, without cell wall (instead of a 3-layer membrane), pleomorphic, phloem dependent only (not reside in the meristem tissues), and have the smallest genome compared to other prokaryotes. The genome has a low guanine and cytosine content (23%, the minimum threshold required for almost vitality). Phylogenetic analysis suggests that phytoplasmas are phylogenetically associated with *Acheloplasma laidlawaii* because they have UGG in place of UGA triplet for tryptophan in other prokaryotes (Bertaccini and Duduk, 2009).

Phytoplasma infection changes metabolic, physiological, and gene expression pathways that result in morphological abnormalities in plants affected. Studies have shown that the presence of phytoplasmas increases phenolic compounds, the production of defense proteins, and hydrogen peroxidase (Junquera et al., 2004). It also impairs iron transport in phloem and causes sugar accumulation in the leaves, causing yellowing symptom and affects the functioning of genes responsible for photosynthesis and flower development, and interferes with plant growth regulators (Buoso et al., 2019; Pracros et al., 2006; Kakizawa et al., 2001).

Depending on the interaction between the pathogen and plant species, symptoms induced by phytoplasmas are considerably typical including yellowing, dwarfing, curly and purplish leaves, dehydrated and hard fruit, witches' broom, big bud, virescence, hypertrophied sepal, impaired blooming, and floral anomalies and sterility (Hoshi et al., 2009; Duduk and Bertaccini, 2011; Giorno et al., 2013).

Phytoplasmas cause destructive damage to more than 700 plant species around the world and are considered as economically restricting factor in many cultivated plants of high agricultural value (Bertaccini et al., 2014; Maejima et al., 2014). Phytoplasmas characteristically cause witches' broom, proliferation in shooting and rooting, flower defects, and increase in the host's metabolic activities by altering phytohormones activity (Musetti et al., 2007; Giorno et al., 2013; Bertaccini et al, 2014). Phytoplasmas have a broad host range and have been reported in many plant species such as ornamental plants, vegetable, grapevines, and fruit tree throughout the world (Seemüller et al., 2002; Alp et al., 2016; Dermastia et al., 2017; Lee *et al*., 2000). Phytoplasma related infections have been reported from ecological zones in Turkey. Phytoplasma presences in different 16Sr groups have been detected from cultivated plants, weeds, fruit and timber trees including onion, potato, tomato and bindweed (Sertkaya et al., 2013; Usta et al., 2018; Ergüven, 2019), olive (Fidan et al., 2012), apple (Sertkaya et al., 2011), pear (Gazel et al., 2007), grapevine (Yurttaş, 2019), pomegranate (Gazel et al., 2015), cherry (Karapınar, 2018), weeping willow (Ul Hassan, 2018), pepper (Mezreli, 2019), and garlic (Danış, 2018).

Although tomato-related phytoplasma diseases have been reported in some regions of Turkey, no information-associated with the phytoplasma disease on tomato in Iğdır province is available. In this study, we attempted to determine the presence of phytoplasma, and its molecular characterization, group and subgroup depending on the 16S rRNA gene in tomato plants exhibiting phytoplasma-type symptoms collected from the Iğdır province of Turkey.

Materials and Methods

Plant Source and DNA Isolation

Four symptomatic tomato plants, as well as symtompless 2 samples, were sampled in the tomato-growing fields in the central Iğdır province, 2019. The samples were placed in plastic bags and transported to the Plant Protection Laboratory of Van Yuzuncu Yıl University in the cold chain for DNA isolation, detection, and molecular evaluation. Total DNA was isolated from 0.5 g frozen leaves tissues (−20°C) using the isolation kit (ISOLATE II Genomic DNA Kit, Germany) according to the manufacturer's instructions. A total of 100 µl eluted DNA was stored at -80°C until use.

Detection of Phytoplasma in Field-İnfected Tomatoes

DNAs obtained from whole leaf of tomato plants grown in open field conditions were tested by two-step PCR (Polymerase Chain Reaction) procedures; direct-PCR (d-PCR) and nested-PCR (n-PCR), highly efficient and

sensitive technique for the phytoplasma identification. To identify the 16S rRNA gene sequence and achieve the amplification, two sets of universal primers (R16mF2/R16mR1 and R16F2n/R16R2) pairs were synthesized as mentioned by Lee et al. (1993) and Gundersen and Lee (1996) for first and second reaction step, respectively. The cycling conditions and PCR reagents were set up as proposed by Lee et al. (1993). Direct and Nested PCR were performed using Eppendorf Mastercycler device (Germany). Direct and Nested PCR assays were performed using Eppendorf Mastercycler (Germany). The reaction mixture (50 µl) contained 5 µl of 10x PCR buffer, 3 µl of 25 mM $MgCl_2$, 1 µl of of 10 mM dNTP Mix, 1 µl of each primer, 5 µl of extracted DNA, 0.4 µl of Go Taq Green polymerase (0.5 U) (Promega, USA) and 33.6 µl of Nuclease free water. The reaction program was 2 min for an initial denaturation step at 94°C following 1 min of denaturation at 94°C, annealing for 2 min at 55°C, extension for 3 min at 72°C for 35 cycles, and a final extension at 72°C for 10 min. The resulted d-PCR products were diluted 30-fold and used as template DNA to n-PCR. Following n-PCR, amplified DNAs of the predicted size (15 µl) were electrophoresed (at 120 for 50 min) using 1.5% agarose gel added the ethidium bromide, analysed with a standard ladder (3000 bp), and then monitored under UV light in gel documentation device. During the PCR tests, an isolate (accession no KJ957010) found in our previous publication (Alp et al., 2016) was used as the positive control. PCR master mix without DNA was used as a negative control.

Cloning, Sequencing, and Similarity Coefficient

A strong PCR positive band selected was gel-purified by DNA isolation kit following the supplier's specifications (ISOLATE II PCR and Gel Kit, BIOLINE) and employed as material in the later stages of the studies. This fragment was ligated into a prokaryotic cloning vector (pGEM T-Easy vector, Promega) using T4 ligation enzyme followed by transferred into competent cells (*E. coli* JM109 strain) using micropulser. A transformed clone that confirmed positive by colony PCR tests was selected and subsequently sequenced by NGS (Sentebiolab/Ankara) after plasmid purification from bacterial cells (ISOLATE II Plasmid Mini Kit, BIOLINE). Plasmids containing 16S rRNA inserts were stored at -80°C for further use. 16S rRNA Iğdır sequence associated with phytoplasma was trimmed from recombinant vector sequences with UGENE program and used for further analysis during the study. Pathogenic location and sequence identity of Iğdır sequence were determined by using the BLASTN search program on the NCBI site, and the similarity coefficient was calculated by web-supported iPhyclassifier software, a useful tool for the assignment of phytoplasma strains.

Consensus Tree and V- RFLP Analyses

To determine the phylogenetic relations and consensus analysis of Iğdır 10 isolate generated from the 1251 nt long 16S rRNA gene was conducted by the MEGA 7 using 19 different members of 16sr group downloaded in GenBank from a different location and hosts (Table 1). For better consensus, *Acholeplasma laidlawii* (accession No. M23932) was chosen as the outgroup. The evolutionary distances were estimated using the neighbor-joining algorithm. 1000 bootstraps scores were used for the robustness of the trees. The bootstrap score percents are given at key nodes.

To determine the structural diversity, the V-RFLP analysis based on the 16S rRNA genomic nucleotide sequence was carried out by pDRAW32 software using seventeen restriction endonuclease enzymes as described by Lee et al. (1998). The obtained V-RFLP gel pattern was compared to that of reference isolate (AY390261, 16SrVI-A) (Hiruki and Wang, 2004).

Results

Symptomatology and Detection of Pathogen By N-PCR

During the studies in 2019, the visual assessment for the phytoplasma-like disease was surveyed in Iğdır province (Turkey). As shown in Figure 1, the phytoplasmic symptoms viz., mosaic, flower infertility, and phyllodes symptoms were observed in tomato plants in fields inspected. Leaf samples associated with phytoplasma were screened for the existence of phytoplasma agents.

The presence of the phytoplasma 16S rRNA gene in symptomatic samples was confirmed by the n-PCR test. The PCR products primed R16F2n/R16R2 amplified typical 1.25 kb DNA fragment, equivalent to the 16S rRNA gene of the phytoplasma (Figure 2). Amongst 6 samples of tomato analysed, n-PCR amplification products of 2 conspicuous symptomatic plant samples were determined to be positive for phytoplasma. No band was observed from healthy plants and negative control.

Sequence Similarity and Coefficient

The sequencing of amplified products raised from the symptomatic tomato plant showed that the 16S rRNA sequence of the Iğdır 10 isolate contained 1251 nucleotides. According to the BLASTN program, the sequence data of the 16S rRNA gene of Iğdır isolate possesses high sequence homology to sequences of phytoplasma members from distinct origins, with ranged from 99.36% to 99.84% nucleotide sequence similarity. The similarity coefficient was estimated as 1.00 by the *i*Phyclassifier software.

V-RFLP Analysis and Consensus Tree

The endonuclease methodologies of interest gene demonstrated that the V-RFLP profile of the Iğdır tomato phytoplasma sequence is closely related to the reference isolate (AY390261, 16SrVI-A group) (Figure 3).

The consensus tree also supported that the species of phytoplasma in infected tomatoes is the 'Ca. P. trifolii'. As shown in Figure 4, the 'Ca. P. trifolii'-related isolates came together in a distinctly different group, including five closely related identical sequences of same strains from tomato, clover, and cucumber and reference sequences (Figure 4).

The phylogenetic and computational analysis associated with Iğdır isolate supported each other. Both analyisis of 16S rRNA gene nucleotide sequence showed that Iğdır phytoplasma isote in tomato is a member of subgroup A in Clover proliferation group (16SrVI-A group). Turkish Iğdır isolate is highlighted by the red dot.

Figure 1. Symptoms related to '*Ca*. P. trifolii' disease in the infected tomato plant. Panel A: upward leaf rolling, foliar purplish, smaller foliar lamina, and bushy shoots. Panel B: defective and abnormal flowers, and elongated sepals.

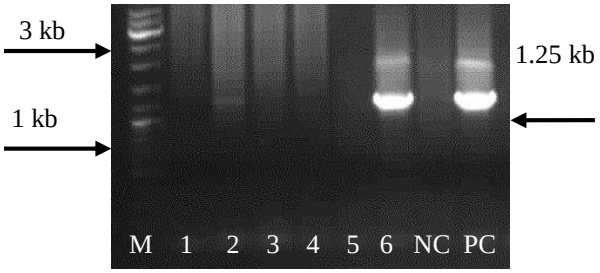

Figure 2. Electropherogram obtained from the n-PCR assays applied to the phytoplasma-symptomatic tomato samples taken from the Iğdır province. M: 1 kb standard DNA ladder; Lane 6: Infected tomato samples; Lane 1, 2, 3, 4, 5: Healthy plants; NC: Negative control; PC: Positive control.

'*Ca*. P. trifolii' "Clover Proliferation" 16SrVI-A group (AY390261) Represantative strain

'*Ca*. P. trifolii' Iğdır 10 isolate (MT344968) Similarity coefficient:1.00

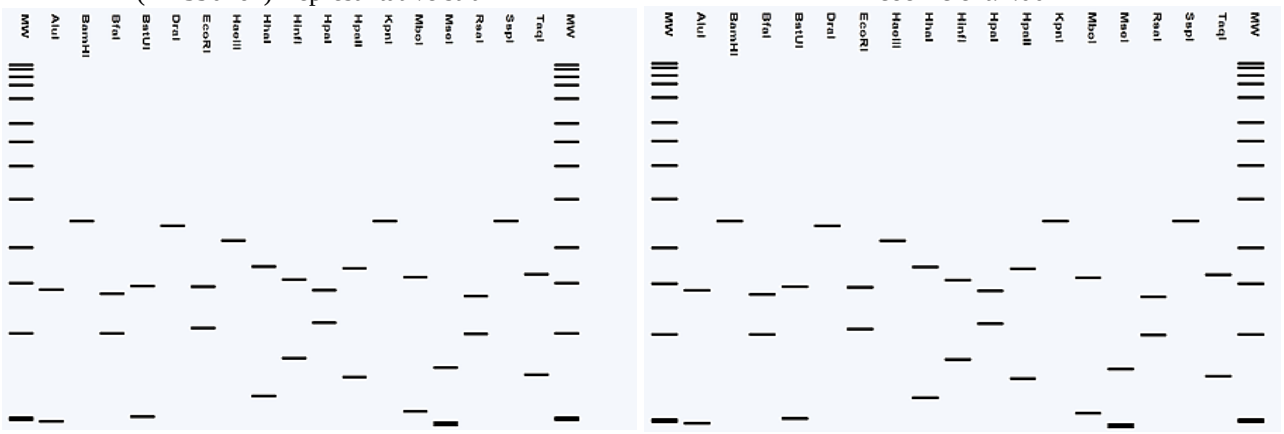

Figure 3. Virtual-RFLP images constructed by pDRAW32 program software using 17 digestion enzymes of 16SrRNA genes of phytoplasma isolate infecting tomato.

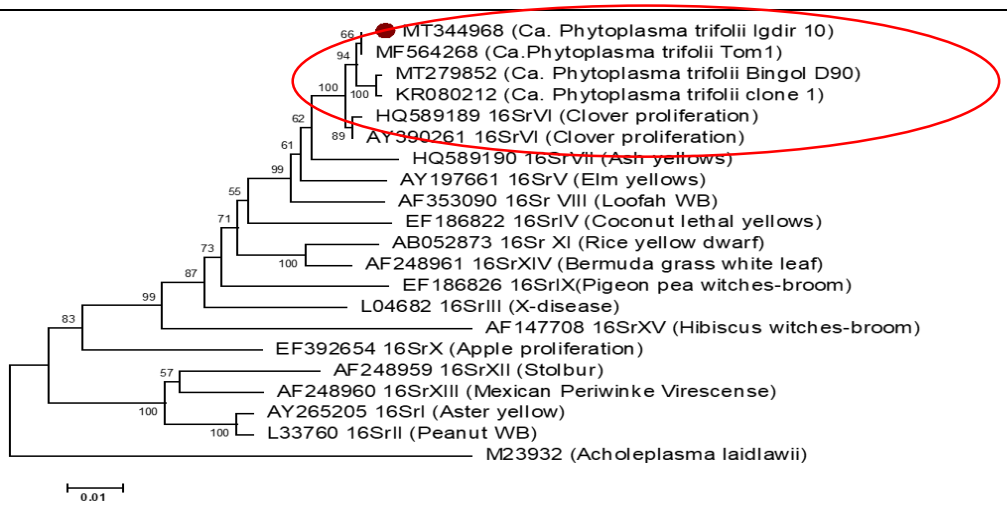

Figure 4. Neighbor-joining relationship dendrogram of the 16s rRNA nucleotide devised with the '*Ca*. P. trifolii' isolate in this study (MT344968), identical sequences of same pathogens, and phytoplasma strains from distinct 16sr groups. Bootstrap values (1000 resamplings) are given on branches of the phylogenetic tree

Discussion

Phytoplasmas were first defined as mycoplasma-like organisms (MLO) in 1967 and called as 'Candidatus' since 2004, which meaning unculturable (Doi et al., 1967; IRPCM, 2004). Phytoplasma infections have been present in Turkey for more than 60 years and distinct phytoplasma strains were identified in various hosts (Tanrıkut, 1953). Phytoplasmas are regarded as one of the main pathogens responsible for the loss of important products in tomato crops. Tomatoes are often infected with various phytoplasma strains from the 16Sr groups, causing fruit defects and abnormal morphology, which leads to huge losses in product and quality.

Table 1. Accession number, location, isolate name and host of phytoplasma strains downloaded from NCBI employed for consensus tree in this work

No	Accession no.	Size (bp)	Location	Isolate name	Host
1	MT344968	1251	Turkey-Iğdır	Iğdır 10	*Lycopersicum esculentum*
2	MF564268	1250	Turkey-Van	Tom1	*Lycopersicum esculentum*
3	MT279852	1250	Turkey-Bingöl	Bingol D90	*Lycopersicum esculentum*
4	KR080212	1251	Turkey-Van	-	*Cucumis sativus*
5	HQ589189	1688	Canada	CP-1	Clover
6	AY390261	1809	Canada: Alberta	CP	*Trifolium hybridum*
7	HQ589190	1785	USA	ASHY-7	*Fraxinus americana*
8	AY197661	1527	-	JWB	Periwinkle
9	AF353090	1760	Taiwan	LfWB	Loofah
10	EF186822	1527	Jamaica	LYja	Periwinkle
11	AB052873	1360	Thailand	RYD-Th	Rice
12	AF248961	1827	Thailand	BGWL	Bermuda grass
13	EF186826	1529	USA: California	PPWBfl	-
14	L04682	1525	-	-	-
15	AF147708	1808	Rio de Janeiro	HibWB26	*H. rosa-sinensis*
16	EF392654	1784	Italy: Valle d'Aosta	T-3	*Malus domestica*
17	AF248959	1783	-	STOL	*Catharanthus roseus*
18	AF248960	1785	-	MPV	*Catharanthus roseus*
19	AY265205	1526	-	IOWB	*Ipomoea obscura*
20	L33760	1476	-	-	*Catharanthus roseus*
21	M23932	1508		Acholeplasma laidlawii	

The principal phytoplasma groups infecting tomato are in group 16SrI, 16SrII, 16SrIII, 16SrV, and 16SrVI (Zamora et al., 2014; Singh et al., 2012; Amaral-Mello et al., 2006; Del Serrone et al., 2001; Anfoka et al., 2003), which induce mostly similar symptoms on their respective host (Krawczyk et al., 2010; Pracros et al., 2006).

About 60 years ago, the Clover proliferation group (16SrVI) was first delineated as a yellow stripe virus in Canada in the *Trifolium hybridum* (Clover) (Chiykowski, 1965). But further characterizations on the 16S rRNA gene and the 16S–23S spacer region carried out by Hiruki and Wang, 2004 indicated that it is a representative of a new species of phytoplasma as '*Candidatus* Phytoplasma trifolii', registered in the GenBank with the accession number AY390261, within Clover proliferation group.

Phytoplasmas cause morphologically dramatic symptoms in the particularly in the member of family Solanaceae. The '*Ca*. P. trifolii' detected in our study induced the big bud, witches' broom, a little and purplish leaves, and growth retardation symptoms in the infected-tomato plant. Observed symptoms are in accordance with the symptoms in phytoplasma-disease tomatoes described by Usta et al. (2018) and Anfoka et al. (2003)

Nowadays, the identification and characterization of phytoplasmas are commonly achieved using the 16S rRNA gene sequence (IRPCM, 2004) besides another indicator gene such as *imp*, *tuf*, *rp*, *cpn60*, *secY*, *rpoB*, and *SAP11* for the taxonomy of phytoplasma in-depth (Alvarez et al., 2014; Davis et al., 2013; Pérez-López et al., 2014; Al-Subhi et al., 2017). Like Hiruki and Wang (2004), we also found the presence of '*Ca*. P. trifolii' isolate using the 16S rRNA gene amplified by d-PCR and n-PCR in the phytoplasma-symptomatic tomato plant. Plus, group/subgroup discrimination of phytoplasma strain associate with Iğdır province was achieved by cloning, sequencing, and RFLP and phylogenetic analysis, as previous researchers have widely adopted. When all analyses were combined, it was determined that the Igdir isolate in infested tomato was '*Ca*. P. trifolii', in the subgroup of Clover proliferation (16srVI group) and A subgroup. The obtained sequence was called 'Iğdır 10' isolate and recorded to the GenBank (MT344968).

Recently, the occurrence of '*Ca*. P. trifolii' has extensively been reported from diverse hosts such as weeds, agricultural and industrial crops including tomato, pepper, grapevine, soybean, safflower, cabbage, maize, sesame, periwinkle, eggplant, American Elm (*Ulmus americana*), Norfolk Island pine (*Araucaria heterophylla*), willow, rapeseed (*Brassica napus*), *Suaeda aegyptiaca*, *Erigeron canadensis*, and *Sorghum halepense*. Current literature screens have shown that infective pathogen has a wider distribution in Asian, European and American countries such as primarily Turkey and Iran, followed by Jordan, Mexico, USA, China, India, and Italy (Reveles-Torres et al., 2018; Shahryari et al., 2019; Ghayeb Zamharir and Aldaghi, 2018; Salehi et al., 2008; Davoodi et al., 2019; Oksal et al., 2017; Ulubaş Serçe and Yılmaz, 2019; Özdemir, 2017; Sertkaya et al., 2007; Flower et al., 2018; Zhang et al., 2012; Zambon et al., 2018; Seyahooei et al., 2017; Amiri Mazraie et al., 2018; Zibadoost et al., 2016; Gupta et al., 2010; Zibadoost and Rastgou, 2016).

The 16S rRNA gene of the Igdir 10 isolate (MT344968) was aligned with that of the reference '*Ca*. P. trifolii' strain (AY390261). Both sequences showed a high degree of consensus with each other, with minor differences including an insertion (T) at position 845, and 5 substitution involving Adenine (A) instead of Guanin (G) at 7 positions, Cytosine (C) instead of Timin (T) at 8 positions, Guanin (G) instead of Adenine (A) at 114 positions, Timin (T) instead of Cytosine (C) at 478 positions, and Adenine (A) instead of Guanin (G) at 994 positions.

Phytoplasmas are evolutionarily successful bacteria. Considering that the adaptation ability of phytoplasmas to

a wide host range, contains more than thirty groups, and newly emerging strains throughout the world, it can be said that sequence differences detected in matching processes are likely to be an essential part of the ongoing evolution of phytoplasmas to acquire new hosts (Davis et al., 2017; Fugita et al., 2017).

Phytoplasmas can overwinter in insect vectors, one-year or perennial plants, which are hosts. Today, a known full-effect treatment application related to phytoplasmas is unknown. Various antibiotics have been tried in previous studies, but they were abandoned due to both their cost and sustainability. In this context, priority should be given to vector control (such as insect and weed) to ensure adequate control for phytoplasmas disease.

Conclusion

Many records present in the literature related to phytoplasma infections in tomato plants. In current work, "*Ca*. P. trifolii" isolate in infected tomato was detected and characterized by online BLASTN program, consensus tree, and virtual RFLP analysis. Result described here is the first report of 16SrVI-A group (Clover proliferation) tomato-phytoplasma infection in Iğdır province of Turkey. In Iğdır province, extensive surveys are needed to determine the prevalence of phytoplasma infection in vegetable fields and orchards, which are important in an economic sense.

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