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Full Length Article

Detection and Molecular Characterization of two '*Candidatus* Phytoplasma Trifolii' Isolates Infecting Peppers at the Same Ecological Niche

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Abstract

Pepper (*Capsicum annuum* L.) cultivars exhibiting phytoplasma like symptoms including yellowing, flower sterility, necrosis, stunting and small leaves of lateral shoots were collected in Spring 2016 from Malatya province (Turkey). Leaf samples of the most common annual weeds and leafhoppers nearby symptomatic peppers were also sampled. Nested-PCR and virtual computer-simulated restriction fragment length polymorphism (virtual RFLP) methods have been implemented to ascertain and characterize the phytoplasma-associated disease. Using universal primer pairs in nested-PCR DNA fragments of approximately 1.2 kb were amplified from 3 pepper samples. None of the weed and leafhopper samples were reacted positive in PCR reactions. Next-generation sequencing (NGS) was used to sequence the amplified PCR fragments of two samples. The presence of '*Candidatus* Phytoplasma trifolii' infections were confirmed by the analysis of 16S rDNA sequence and virtual-RFLP. Molecularly characterized isolates were designated as TR1 and TR2 (Acces. no: KY321932 and KY568694). Both isolates were identified as members of the clover proliferation phytoplasma group (subgroup 16SrVI-A) in pepper plants as strains of '*Ca.* Phytoplasma trifolii'. Sequence alignment of the two '*Ca*. Phytoplasma trifolii' isolates revealed a low level of genetic diversity. However, the restriction enzyme patterns of both isolates particularly in *Mse*I profiles were differed from reference patterns of all previously established '*Ca.* Phytoplasma trifolii' isolates in the world. Particularly, the TR2 isolate showed a point mutation comparing TR1 isolate and with reference strain (AY390261) of '*Ca*. Phytoplasma trifolii'. This is the first report of '*Ca*. Phytoplasma trifolii' isolates naturally infecting pepper plants from Turkey. © 2017 Friends Science Publishers

Keywords: Pepper; '*Ca.* phytoplasma trifolii'; Molecular characterization; Turkey

Introduction

Phytoplasmas are phloem-limited cell wall-less bacteria that are insect transmitted pathogenic agents usually causing flowering and growth abnormalities, together with witches' brooms, phyllody, chlorosis and stunting in their hosts (Davis *et al*., 2006). They are non-cultivable degenerate gram-positive prokaryotes and are considered amongst the smallest self-replicating living organisms having very small genome sizes (Bertaccini *et al*., 2014).

Phytoplasmas infect numerous important crop plants resulting significant yield losses including peppers and other Solanaceous crops. Infection of peppers have been reported from several areas of the world (Cousin *et al*., 1989; Fos *et al*., 1992; Avinent and Llacer, 1995; Marcone and Ragozzino, 1995).

Previously, 16SrVI Clover proliferation (CP) group of phytoplasmas were described associated with a disease of alsike clover in Canada (Chiykowski, 1965a). Based on subsequent investigations today, it is taxonomically classified as '*Candidatus* Phytoplasma trifolii' (Hiruki and Wang, 2004). Recently, the agent was reported in tomato and pepper in Jordan and Spain, respectively (Castro and Romero, 2002; Anfoka *et al*., 2003).

Phytoplasma associated diseases of pepper have been reported in all main pepper-growing areas worldwide (Pracros *et al*., 2006; El-Banna *et al*., 2007; Özdemir *et al*., 2009). In Turkey, phytoplasma infections occured in numerous cultivated crops such as pepper, tomato, sesame and eggplant (Alp *et al*., 2016). '*Ca.* Phytoplasma trifolii' phytoplasma was previously found to be associated with sesame phyllody disease in sesame in Turkey (Sertkaya *et al*., 2007).

Phytoplasmas are known to be transmitted by leafhoppers belonging to Cicadellidae family. Reproduction in their vectors have been demonstrated for several species (Ploaie, 1981). It has been reported that more than one vector may transmit the same phytoplasma and more than one phytoplasma isolate may be transmitted by the same leafhopper species (Chiykowski, 1965b; Bosco *et al*., 1997).

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The utility of PCR in amplification of 16S rRNA gene and *in silico* restriction fragment length polymorphism (RLFP) analysis have been reported effective in detection, differentiation and characterization of phytoplasmas (Deng and Hiruki, 1991; Ahrens and Semüller, 1992; Wei *et al*., 2007).

During field surveys in 2016, pepper plants showing flower sterility, yellowing, necrosis, small leaves of lateral shoots and stunting were observed in Malatya province, Turkey. To identify and classify the relevant phytoplasma-associated agents the biological and molecular characteristics were investigated. Here we report the occurrence of '*Ca.* Phytoplasma trifolii' (16SrVI group) in pepper plants in Turkey and phylogenetic relationships among strains in the 16SrVI group phytoplasma using sequences from the 16S rRNA gene. To the authors' knowledge this is the first record of *Ca*. Phytoplasma trifolii infecting pepper plants in Turkey.

Materials and Methods

Collecting, Pepper, Weed and Insect Samples and DNA Extraction

Pepper plants with typical phytoplasma symptoms and without symptoms were sampled at the beginning of growing season in 2016. Symptomatic plants identified in the field were inspected from May to September. In addition, the leaf samples of the most common annual weeds and possible insect vectors such as leafhoppers nearby the symptomatic pepper plants were sampled. In August 2016, adults of *Emposca decipiens* and *Zyginidia pullula* were captured on symptomatic pepper plants by sweep net sampling. Collected adult insects were stored in 96% ethanol until DNA extraction. The all collected materials were shipped to laboratory in a cool chain for phytoplasma testing. Fresh leaf tissues were used in the extraction of genomic total DNA by using a commercial genomic DNA purification kit (Jena Biosciences, Germany). DNA from an asymptomatic pepper plant was served as the negative control. In diagnosis of the agent by PCR assay an isolate of '*Ca*. Phytoplasma solani' identified positively from preliminary tests was used as positive control.

Detection of Phytoplasma and '*Ca*. Phytoplasma trifolii'

R16mF2/R16mR1 and R16F2n/R16R2 universal primers (Lee *et al*., 1993; Gundersen and Lee, 1996) were used for amplification, detection and characterization of possible phytoplasma agents in samples prepared from pepper, weed and insect tissues by Nested polymerase chain reaction (Nested-PCR) method. PCR reactions were performed in a Thermo Scientific Arktik Thermal Cycler.

A 50 µL of PCR reaction volume was contained 1 µL of sample DNA, 5.0 µL of 5 x PCR reaction buffer, 1.5 mM $MgCl_2$, 1.0 µL of dNTP mixture (each at 2.5 mM), 0.25 µL of each primer and 0.5 U of *Taq* DNA polymerase enzyme (Jena Biosciences, Germany). The temperature program consisted with 2 min for an initial denaturation step at 94°C followed by 35 cycles of 1 min denaturation at 94°C, 2 min annealing at 55°C and 3 min extension at 72 °C and a final extension at 72°C for 10 min (Lee *et al.*, 1993). Before visualizing with a UV trans-illuminator the amplified DNA was electrophoresed on 1.5% agarose gel and stained with Pronasafe nucleic acid staining solution (CondaLab).

DNA Sequencing of Amplified PCR Products, Sequence and Phylogenetic Analysis

The PCR products (about 1.2 kbp) were purified by using a PCR purification kit (Jena Biosciences, Germany) per the manufacturers' instructions. Nucleotide sequences were submitted in GenBank. DNA sequences were blasted against GenBank database in the National Center for Biotechnology Information (NCBI). Based on 16S rRNA gene sequences the phylogenetic interrelationships among strains of Clover proliferation group and other phytoplasma groups were assessed. Partial sequences of 16S rDNA from members of the 16SrVI group and other phytoplasma strains that were available in GenBank were aligned by using CLC Main Workbench Software version 6.2.2. A phylogenetic tree of 16S rDNA sequences was constructed using CLC Main Workbench Software by 1000 replicates.

In Silico Digestion of 16SrDNA Sequences

A virtual gel plotting software, pDRAW32 (AcaClone Software) was used to obtain computer simulated patterns of the virtual restriction fragment length polymorphism (RFLP) of the 16S rDNA sequences. To obtain *in silico* RFLP digestion patterns of each 16S rDNA fragment (Lee *et al*., 1998), seventeen distinct restriction endonucleases including *Alu*I, *BamH*I, *Bfa*I, *Bst*UI (*Tha*I), *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hinf*I, *Hpa*I, *Hpa*II, *Kpn*I, *Sau3*AI (*Mbo*I), *Mse*I, *Rsa*I, *Ssp*I and *Taq*I were used in computer simulated 1.0% agarose gel electrophoresis. The virtual gel images were then captured on computer screen. For calculation of similarity coefficient and RFLP pattern comparisons the 16Sr DNA sequences were analyzed using web based i*Phy*Classifier (Zhao *et al.*, 2009) program and pDRAW32 software.

Results

A total of 17 pepper, weed and insect samples were collected and tested against possible phytoplasma diseases from Malatya province (Turkey) in 2016. The samples included 4 pepper plants, 4 weeds and 9 leafhoppers. Using R16mF2/R16mR1 and R16F2n/R16R2 universal primers, phytoplasmas associated with yellowing, flower sterility, necrosis, stunting and small leaves of lateral shoots of

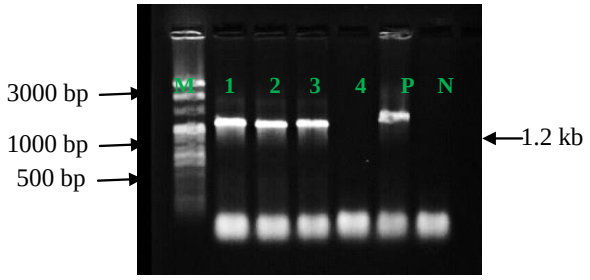

Fig. 1: Nested-polymerase chain reaction (PCR) detection of '*Ca*. Phytoplasma trifolii' using universal primer pairs. The rows 1-4 are tested pepper samples, rows 1-3 are positively reacted pepper samples, M: 10.000 bp molecular markers, N: negative control, P. positive control

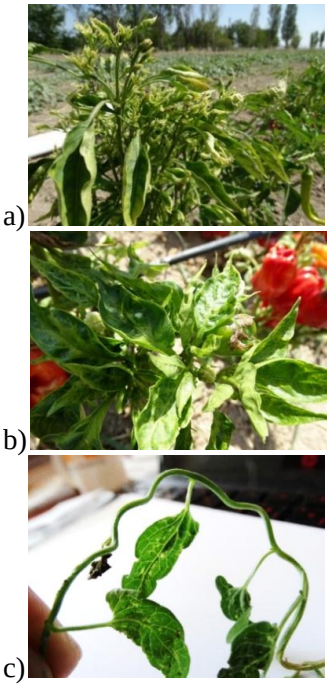

Fig. 2: Symptomatic plants identified in the field were inspected from May to September. Pepper (*Capsicum annuum* L.) plants exhibiting yellowing, flower sterility, necrosis, stunting and small leaves of lateral shoots (a) in Spring 2016 and leaf mosaic, vein banding and leaf deformation in the late summer of the same year (b), *C. arvensis* showing leaf deformation and vain banding symptoms (c)

pepper plants were detected by nested-PCR in 3 out of 4 pepper samples. Typical bands, specific to phytoplasma from peppers (approx. 1.2 kb) and the positive control were visualized in agarose gel as shown in Fig. 1. No amplicon was observed when DNA from asymptomatic plant was used as a template in the negative control. Based sequencing data, two isolates of '*Ca*. Phytoplasma trifolii' (16Sr VI-A group) were ascertained in peppers and designated as TR1 and TR2 isolates.

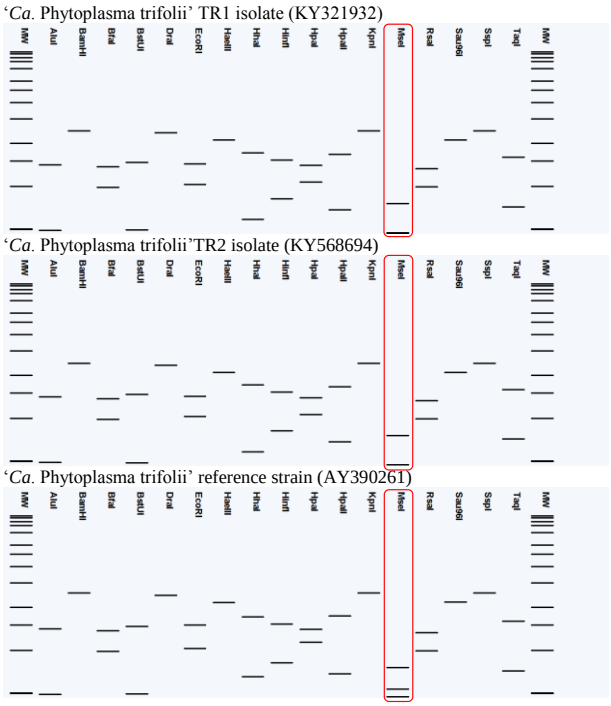

Fig. 3: Analysis of virtual R16F2n/R2 RFLP profiles of '*Ca*. Phytoplasma trifolii' TR1 and TR2 isolates by key enzyme *Mse*I. The restriction profiles of *Mse*I showed a distinct pattern separating the Turkish isolates from '*Ca*. Phytoplasma trifolii' reference strain (AY390261) (indicated in boxes), MW, 1kb Promega size markers

The tested weeds were included: *Amaranthus retroflexus* L., *Anthemis arvensis*, *Convolvulus arvensis* L., and *Sorghum halepense* (L.) Pers. No visual symptoms were observed in weed samples except *C. arvensis* which exhibited leaf deformation and vain banding symptoms (Fig. 2c). However, no phytoplasma was detected in weed and insect samples collected from pepper field. The adults of *Emposca decipiens* and *Zyginidia pullula* (Boheman, 1845) (Homoptera: Cicadellidae) were captured on symptomatic pepper plants by sweep net sampling. The all tested *E. decipiens* and *Z. pullula* samples were reacted negative in nested-PCR assays.

The PCR products of the same size (approx. 1250 bp) were obtained in nested PCR from all positively reacted samples. Randomly selected two F2nR2 fragments of 16S rDNA were sequenced and digested with 17 restriction enzymes. *In silico* RFLP analysis revealed variability among TR isolates and comparing with the reference strain in *Mse*I profiles (Fig. 1). The restriction profiles of TR1, TR2 and reference strain are shown in Fig. 3.

Virtual RFLP analyses of 16S rDNA F2nR2 fragment of pepper phytoplasmas (Acces. no: KY321932 and KY568694) with 17 restriction enzymes resulted identical

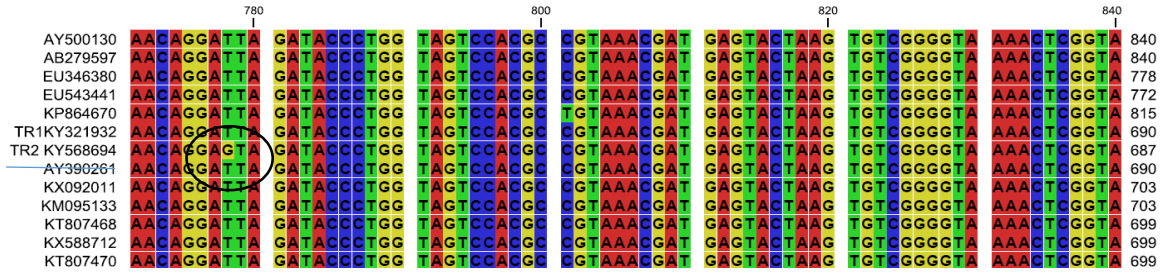

Fig. 4: Sequence alignment of Turkish '*Ca*. Phytoplasma trifolii' isolates (TR1 and TR2) with available world isolates of same species

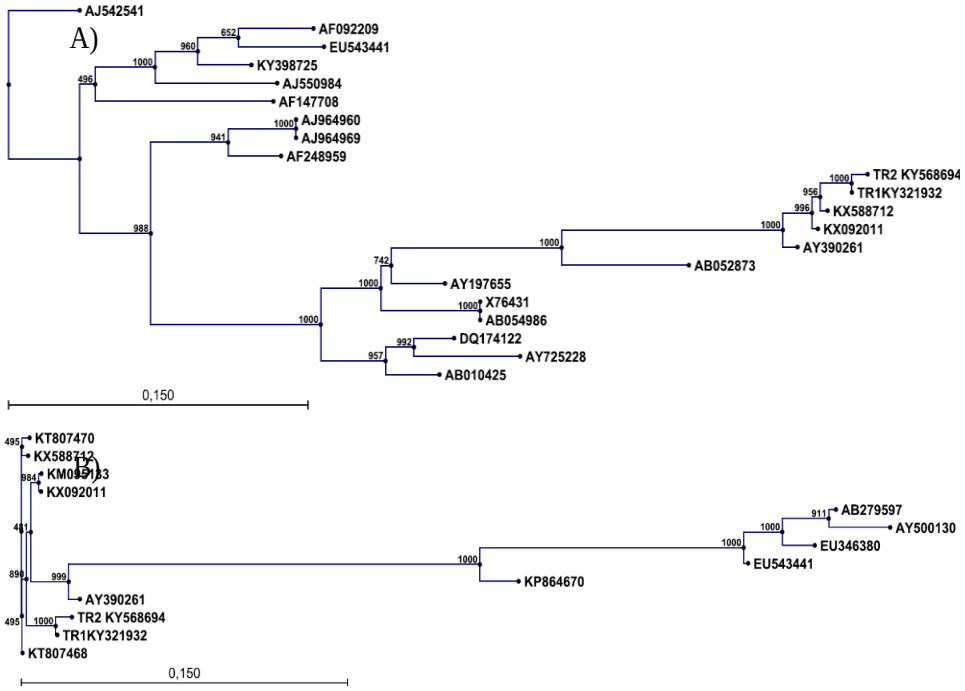

Fig. 5: Phylogenetic tree of 16Sr-DNA sequences of '*Ca*. P. trifolii' TR1 and TR2 isolates constructed with neighbor joining algorithm using the reference strain and other world '*Ca*. Phytoplasma' species isolates (panel A) and other world '*Ca*. Phytoplasma trifolii' isolates (panel A). The bootstrap values (1000 replicates) are shown on branches. Sequences for '*Ca*. Phytoplasma' species were retrieved from NCBI Genbank (KT807470, FX588712, KM095133, KX092011, AB279597, AY500130, EU346380, EU543441, KP864670 and KT807468), '*Ca*. P. trifolii' TR1 isolate; KY321932 (this publication), '*Ca*. P. trifolii' TR2 isolate; KY568694 (this publication), '*Ca*. P. trifolii; AY390261 (Hiruki and Wang, 2004), '*Ca*. P. trifolii; KX588712 (Das *et al*., 2016), '*Ca*. P. trifolii; EU543441 (Přibylová *et al*., 2008), '*Ca*. P. trifolii; KX092011 (Muñoz *et al*., 2016), '*Ca*. P. mali'; AJ542541 (Seemüller and Schneider, 2004), Western X-disease phytoplasma L04682; (GenBank submission, 1999), '*Ca*. P. fraxini'; AF092209 (Griffiths *et al*., 1999), '*Ca*. P. oryzae; AB052873 (Jung *et al*., 2003), '*Ca*. P. solani'; AJ964960 (Firrao *et al*., 2005), '*Ca*. P. japonicum'; AB010425 (Sawayanagi *et al*., 1999), '*Ca*. P. solani' STOL; AF248959, '*Ca*. P. cynodontis'; AJ550984 (Marcone *et al*., 2004), '*Ca*. P. graminis'; AY725228 (Arocha *et al*., 2005), '*Ca*. P. brasiliense'; AF147708 (Montano *et al*., 2001), '*Ca*. P. castaneae'; AB054986 (Jung *et al*., 2002), '*Ca*. P. americanum'; DQ174122 (Lee *et al*., 2006) and '*Ca*. P. solani'; AJ964960 (Firrao *et al*., 2005)

restriction patterns (with similarity coefficient of 1.00 for both isolate) to the reference pattern of 16Sr group VI, subgroup A. BLAST analysis of the 16S rDNA sequence and virtual RFLP analysis confirmed the presence of the of '*Ca*. Phytoplasma trifolii', with a high similarity comparing with reference phytoplasma isolate (GenBank accession no.: AY390261) (Fig. 3). With the exception of a digestion profile of full-length fragments of R16F2n/R16R2 obtained by *Mse*I digestions both isolates were exhibited identical patterns for all of the other 16 key restriction enzymes. *Mse*I

digestions were produced only two fragments while the reference strain was resulted three bands indicating genetic diversity between Turkish isolates and reference strain of '*Ca*. Phytoplasma trifolii'. As shown in Fig. 3 the isolates were separated only by the *Mse*I digestion profiles from reference strain compared.

Sequencing of PCR Products

Sequence data of 16S rDNA was used to examine the genomic diversity between pathogenic '*Ca*. Phytoplasma trifolii' isolates. The results showed that '*Ca*. Phytoplasma trifolii' TR1 and TR2 isolates isolated from same ecological niche, pepper plants are surprisingly diverse compared to each other and the related sequence of reference strain of the same pathogens. A single-base mutation on the 16S rRNA gene of TR2 isolate was detected (Fig. 4.). The full-length fragments of R16F2n/R16R2 of TR1 isolate was found 8 nucleotides longer than TR2 isolate.

Phylogenetic Analysis

In the phylogenetic analysis the nucleotide sequences of TR1 and TR2 isolates were analyzed. The sequences generally clustered among the isolates of according to phylogeny of '*Ca*. Phytoplasma trifolii'. Phylogenetic analyses of 16S rRNA sequences yielded three clusters which 'Ca. Phytoplasma trifolii' TR1 and TR2 isolates were formed in the same cluster. Among the studied isolates the TR2 isolate was distinguished from the other phytoplasma strains by its phylogenetic position mostly because of the mutation on its rRNA sequence. Turkish isolates of '*Ca*. Phytoplasma trifolii' formed a sub-branch with other '*Ca*. Phytoplasma trifolii' world strains with an identity score of 99% (Fig. 5).

Discussion

Sequence and *in silico* RFLP analyses of '*Ca*. P. trifolii' TR1 and TR2 isolates clearly indicated that both isolates cause pepper phytoplasma disease in Malatya province. Among the tested pepper, weed and insect specimens, '*Ca*. Phytoplasma trifolii' (16Sr VI-A group) isolates were detected only in peppers.

Comparison of R16F2n/R16R2 approx. 1250 bp product sequences derived from two symptomatic pepper samples and the sequences from Gen Bank database indicated 99% similarity with the 16S rRNA gene sequences from '*Ca*. Phytoplasma trifolii'. This clearly indicates the presence of the '*Ca*. Phytoplasma trifolii' infections in symptomatic peppers.

Arthropod vectors of phytoplasmas are mostly planthoppers (Fulgoromorpha), psyllids (Psyllidae) and leafhoppers (Cicadellidae) which are phloem feeders of the Order Hemiptera, (Weintraub and Beanland, 2006). After entomological identification of the specimens as *E. decipiens* and *Z. pullula* nested PCR analysis was performed for the presence of phytoplasma infection. In our experiments, none of the 9 insect samples reacted positive in PCR assays which were collected around the naturally infected pepper plants. However, some *Empoasca* species have been reported as potential phytoplasma vectors. For instance, in Cicadellidae family the presence of pathogen was reported based on PCR assays. In different studies, *E. decipiens* individuals were tested positive in PCR assays for '*Ca.* P. asteris' (16SrI-B) and '*Ca.* P. aurantifolia' (16SrII-B) (Parrella *et al*., 2008; Galetto *et al*., 2011). However, in other studies, *Empoasca* spp. were tested negative in PCR assays those particularly collected from phytoplasma-infected agricultural fields (Sertkaya *et al*., 2007; Duduk *et al*., 2008). None of the tested *Z. pullula* samples were reacted positive in PCR assays. In literature, the leafhopper species *Z. pullula* was not reported as having a role in transmission of phytoplasmas (Drobnjaković *et al.*, 2011).

To date, '*Ca*. Phytoplasma trifolii' has been detected on few weedy hosts including *Datura stramonium* L., *D. inoxia* and Calotropis gigantea (Raj *et al.,* 2009; Priya *et al.,* 2010). The absence of the agent in tested weed samples should be considered that the '*Ca*. Phytoplasma trifolii' possibly has alternative solanaceous surviving hosts for its epidemiology other than the tested weed species in this study around the infected plants.

Wei *et al*. (2007) suggested that 19 of the 28 groups of phytoplasmas could be sufficiently differentiated by *Mse*I digestion profiles alone including subgroup 16SrVI-A phytoplasmas. However, our studies revealed that *Mse*I digestion profiles alone is not sufficient to differentiate the '*Ca*. Phytoplasma trifolii' isolates. This is attributed mostly to genetic diversity of the most isolates which is likely due to substitutions on 16SrRNA gene. The emergence of new variants suggests ongoing evolution in adaptation of the phytoplasmas to a new ecological niche (Arocha-Rosete *et al*., 2011).

The present results confirmed that the association of 'Ca. Phytoplasma trifolii' with pepper plants in the eastern region of Turkey. The actual potential of pepper phytoplasmas in pepper fields of Malatya may be higher than our preliminary survey. Phytoplasma diseases are widespread in many regions of the country, but this research is the first report from association of '*Ca*. Phytoplasma trifolii' with peppers in Turkey. Further surveys may help to expanding the knowledge of the epidemiology and genetic diversity of 16SrVI-A phytoplasmas and to identify and classify the phytoplasma strains in Turkey and worldwide.

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