YYÜ TAR BİL DERG
(YYU J AGR SCI)
2017, 27(3): 299-308

Geliş Tarihi (Received): 30.01.2017
Kabul Tarihi (Accepted): 21.04.2017

Araştırma Makalesi/*Research Article (Original Paper)*

Detection and Characterization of two Phytoplasma lineages on Cucumber (*Cucumis sativus* L.) with Same Symptomatology based on Virtual RFLP and Nucleotide Sequence Analysis of 16S rDNA

Mustafa USTA[1] Abdullah GÜLLER[1] Hikmet Murat SİPAHİOĞLU[2]

[1]Department of Plant Protection, Faculty of Agriculture, Van Yuzuncu Yil University, Van, Turkey
[2]Department of Plant Protection, Faculty of Agriculture, Inonu University, Battalgazi, Malatya, Turkey
* e-posta: mustafausta@yyu.edu.tr

Abstract: Phytoplasma-like symptoms were observed in cucumbers (*Cucumis sativus* L.) in Van province of Turkey. The major symptoms observed were severe dwarfing, witches' broom, rosetting, little leaf, and sterility of plants. Genomic DNA of 8 symptomatic and non-symptomatic plant leaves was isolated for the detection of pathogenic DNA. Of the 8 cucumber leaf samples tested by nested polymerase chain reaction (Nested-PCR), the four yielded the expected 1.25-kb DNA fragments when using universal primer pairs R16mF2/R16mR1 and R16F2n/R16R2. Randomly selected two DNA bands were further cloned into a proper plasmid vector. The recombinant plasmid DNA was sequenced bidirectionally. BLAST and virtual restriction fragment length polymorphism (RFLP) analyses of the 16S rDNA sequence revealed the presence of the "*Candidatus* Phytoplasma solani" (similarity coefficient 1.00) (GenBank accession no: KX977570) in one of the severely symptomatic cucumber samples and the "*Candidatus* Phytoplasma trifolii" (similarity coefficient 0.98) (GenBank accession no.: KR080212) in the other. The isolates were designated as Van-trifolii and Van-solani isolates, respectively. No significant differences were observed between the two different phytoplasmas' symptomatology. To the authors' knowledge, this is the first report of '*Ca.* P. trifolii' and '*Ca.* P. solani' in cucumbers in Turkey.

Keywords: *Candidatus* Phytoplasma solani, *Candidatus* Phytoplasma trifolii, Characterization, *Cucumis sativus*, Detection

Hıyar (Cucumis sativus L.) Bitkisinde Aynı Simptomatolojiye Sahip İki Farklı Fitoplazma etmeninin 16S rDNA'sının RFLP ve Nükleotid Dizi Analizleri ile Teşhisi ve Karakterizasyonu

Özet: Van ilinde yetiştiriciliği yapılan hıyarlarda (*Cucumis sativus* L.) fitoplazma benzeri belirtiler görülmüştür. Gözlenen en önemli belirtiler arasında şiddetli cüceleşme, cadı süpürgesi görünümü, rozetleme ve küçük yapraklılık yer almaktadır. Toplanan 8 hıyar örneğine R16mF2/R16mR1 ve R16F2n/R16R2 primer çiftleri ile uygulanan Nested-PCR testi uygulanmıştır. Belirti gösteren ve göstermeyen hıyar yapraklarından izole edilen genomik DNA'ların kullanıldığı PCR reaksiyonunda fitoplazma etmenlerine ait 16S rDNA fragmentleri çoğaltılmış ve test edilen sekiz örneğin dördünde 1.25 kb uzunluğunda DNA fragmentleri elde edilmiştir. Beklenen band büyüklüğünü veren örneklerden rastgele seçilen iki örnek uygun bir plasmid vektörde klonlanmıştır. Saflaştırılan rekombinant plasmid DNA'sı çift yönlü olarak dizilenmiş ve 16S rDNA dizisinin BLAST ve RFLP analizleri yapılmıştır. Şiddetli fitoplazma belirtisi gösteren hıyar numunelerinin birinde '*Candidatus* Phytoplasma solani' (benzerlik katsayısı 1.00) (GenBank erişim no: KX977570), diğerinde ise '*Candidatus* Phytoplasma trifolii' (benzerlik katsayısı 0.98) (GenBank erişim no: KR080212) saptanmıştır. İzolatlardan birincisine'Van-solani' diğerine ise 'Van-trifolii' izolatı isimleri verilmiştir. İki farklı fitoplazma etmeninin oluşturduğu belirtiler arasında bir fark gözlenmemiştir. Yazarların bilgisine göre, Türkiye'de hıyarları doğal olarak enfekte eden "*Ca*. P. solani" ve "*Ca*. P. trifolii" fitoplazma etmenleri ilk defa bu çalışma ile rapor edilmiştir.

Anahtar kelimeler: *Candidatus* Phytoplasma solani, *Candidatus* Phytoplasma trifolii, Karakterizasyon, *Cucumis sativus*, Survey

Indroduction

Phytoplasmas are plant pathogenic, triple membranous, wall-less *Mollicutes*. They have been reported in many plant varieties of crops and trees of agricultural importance worldwide (Khan et al. 2003). In plants, typical phytoplasma symptoms include: virescence, witches' broom, reddening of leaves, phloem necrosis, dieback, phyllody, stunting, small leaves, yellowing of leaves, sterility and big bud (Davis et al. 1997; Hogenhout et al. 2008). The phytoplasmas can be transmitted by dodders, grafting, and by psyllids or plant hoppers (Khan et al. 2003).

The genetic diversity and evolutionary origin of phytoplasmas are largely unknown. However, recent studies suggest the utility of amplified genomic DNA fragments extracted from phytoplasma infected plants has made detection primers available for detection, identification and their genetic relationships of these pathogens (Ahrens and Seemüller 1992; Namba et al. 1993; Bertaccini et al. 2014). Today, phytoplasma diseases are identified, differentiated and detected on the basis of characteristic symptoms, host range, and lately molecular analysis (Al-Saady et al. 2008). According to a recently adopted taxonomic rule, the properties of uncultured organisms should be recorded by a *Candidatus* designation (Murray and Schleifer 1994; Murray and Stackebrandt 1995; IRPCM 2004). The utility of molecular tools led to the designation of a new taxon named '*Candidatus* phytoplasma' and the use of new trivial name of "phytoplasma". When the biological characteristics are used for classification, the term '*Candidatus*' should be dropped (IRPCM 2004; Duduk and Bertaccini 2011).

Phytoplasma associated symptoms have been observed in many cultivated fruit, vegetable, ornamental and field crops in Turkey (Alp et al. 2016; Başpınar et al. 1993;. Çağlar et al. 2010; Çağlar and Elbeaoino 2013; Özdemir et al. 2009; Özdemir and Saygılı 2012; Sertkaya et al. 2004; Sertkaya et al. 2005; Sertkaya et al. 2007; Sertkaya et al. 2008; Ulubaş Serçe et al. 2006; Gazel et al. 2016). Field cucumber (*Cucumis sativus* L.) has been considered one of the most economically important vegetable crops in Turkey. In recent years, cucumber plants showing rosetting, stunting, phyllody, and sterility symptoms were observed in the eastern part of Turkey. The present study was carried out to identify and characterize the causal phytoplasma (s) which affects the yield of cucumber fields. The results of this study revealed that cucumbers were diseased by two Phytoplasma lineages: "*Candidatus* Phytoplasma trifolii" and "*Candidatus* Phytoplasma solani". This research is the first report on 16SrXII group phytoplasma infection of *C. sativus* in Turkey.

Materials and Methods

Plant material and DNA extraction

Symptomatic leaves of *Cucumis sativus* L. showing dwarfing, pyhllody, rosetting, and sterility were collected from symptomatic and non-symptomatic cucumber plants in 2015 from cucumber fields at Van province (Turkey). Using a commercial DNA extraction kit (Vivantis), the genomic DNA was purified from fresh leaf tissues and midrib of 8 cucumber samples as described by manufacturer. A tomato isolate of '*Ca*. P. solani', identified from preliminary tests, was used as a positive control for diagnosis of the phytoplasmas. Genomic DNA from a healthy cucumber plant was served as negative control.

Detection of 'Ca. P. trifolii' and 'Ca. P. solani' by Nested-PCR

The universal primer set (R16mF2/R16mR1 and R16F2n/R16R2) were employed to detect phytoplasma DNA. Fresh cucumber leaf tissues were used in Nested-PCR to amplify 16S rDNA (Lee et al. 1993; Gundersen and Lee 1996). Nested PCR was performed in 50 µl reaction volume using Eppendorf Mastercycler (Germany). The reaction mixture and temperature regime was employed as described by Alp et al. (2016). The first round PCR products were diluted 100 fold using sterile distilled water PCR tubes and used in nested step as template. The same temperature cycles were used at this step. All PCR products were analyzed on 1% agarose gel using Tris-Borate EDTA (TBE) buffer, stained with ethidium bromide and visualized with an UV transilluminator.

Cloning, sequencing and cladistic analysis

The purified amplicons from polymerase chain reaction were ligated into pGEM-T Easy Vector (Promega, Madison, WI, USA). The recombinant plasmids were then transformed into competent cells (*Escherichia*

coli JM109) by electroporation. Using a commercial miniprep kit (Fermentas), the recombinant plasmids were purified and sequenced directionally. Based on 16S rRNA gene sequences, the phylogenetic relationships among strains of the '*Ca.* Phytoplasma trifolii' and '*Ca.* Phytoplasma solani' and other phytoplasma groups available in NCBI (http://www.ncbi.nlm.nih.gov/gquery/gquery.fcgi) were assessed. The sequences of phytoplasma16S rDNA from NCBI were aligned by using MEGA4 and CLC Main workbench 6.6.1 software and the cladistic analyses were performed by using MEGA4. Uninformative characters were excluded from analyses. The relationships were assessed using 1000 bootstrap replicates. *Acholeplasma laidlawii* was selected as the outgroup to root the phylogenetic tree. The phylogenetic tree was created using Neighbor-Joining method from 16S rDNA sequences of other 22 phytoplasmas representing distinct phytoplasma groups and the phytoplasmas studied in this work.

In silico restriction enzyme digestions and virtual RFLP analysis

In virtual restriction fragment length polymorphism (RFLP) analysis, each DNA fragments of 16S rDNA were digested *in silico* with 17 distinct restriction enzymes: *Alu*I, *Bam*HI, *Bfa*I, *Bst*UI (*Tha*I), *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Sau*3AI (*Mbo*I), *Mse*I, *Rsa*I, *Ssp*I, and *Taq*I (Lee et al. 1998). The 16S rDNA sequences were trimmed in virtual gel plotting program pDARW32 software and web based i*Phy*Classifier software was used to calculate similarity coefficient (Wei et al. 2007). Following the digestion, a virtual 1.0 % agarose gel electrophoresis image was created in computer screen. In virtual RFLP analysis sequences of cucumber phytoplasma isolates were automatically compared with each reference strains for group and subgroup recognition.

Results

Amplification of phytoplasmal 16S rDNA fragments and the RFLP analysis of PCR products of the symptomatic cucumber samples revealed that the cucumber plants were infected by two phytoplasmas: '*Ca.* Phytoplasma trifolii' and '*Ca.* Phytoplasma solani'. The isolates were designated as Van-trifolii and Van-solani, respectively. The sequences of 16S rDNA from both isolates were deposited in GenBank (Accession numbers: KX977570 and KR080212). Under the field conditions the symptoms of '*Ca.* Phytoplasma trifolii' and '*Ca.* Phytoplasma solani' on cucumber were similar. In Figure 1a and 1b are shown diseased cucumber plants with characteristic phytoplasma symptoms of excessive branching of axillary shoots, elongation and etiolation of internodes, downward posture of leaves, phyllody, and general stunting of plants in Van Province.

a)  b) 

Figure 1. Severely stunted cucumber plants showing rosetting infected by '*Ca*. P. trifolii' (a), and exhibiting heavy proliferations of small leaves infected by '*Ca*. P. solani'(b).

Direct and nested PCRs with primer pair R16mF2/R16mR1 and R16F2n/R16R2 yielded approx. 1.25 kb DNA fragments from purified DNA extractions of symptomatic cucumber samples (Figure 2). When the DNA from healthy plants was used as control the same primers did not produce any PCR products.

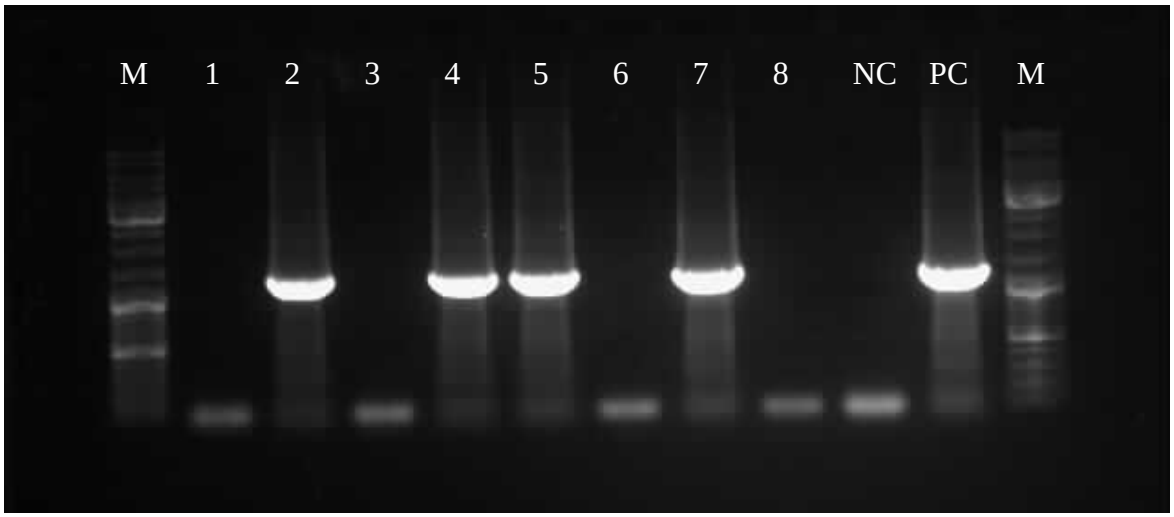


Figure 2. Nested-PCR detection of '*Ca*. P. trifolii' and '*Ca*. P. solani'. Lanes 1–8 are tested fresh cucumber leaf samples, 2,4,5, and 7 (approx. 1.25 kb) are positively reacted samples, NC: negative control of the same species, and P: '*Ca*. P. solani' used as positive control, M:10,000 bp molecular size markers.

A further phylogenetic analysis of 16S rRNA gene sequence was implemented to demonstrate the phytoplasma nature of the infected symptomatic cucumbers. The topology of the phylogenetic tree constructed by MEGA4 program (Tamura et al. 2007) clearly demonstrated that the agents associated with the disease in cucumbers are belong to the phytoplasma clade. The isolates clearly shared common ancestors with '*Ca*. P. trifolii' and '*Ca*. P. solani' (Figure 3). Sequence analysis of isolates confirmed that one of the characterized isolates (Van-solani) in ourstudy belong to the 16SrXII Stolbur group (subgroup XII-A) and the other (Van-trifolii) was belong to the 16SrVI clover proliferation group (subgroup VI-A).

The computer-simulated 16S rRNA gene analysis allowed identification and differentiation of both Turkish isolates by producing virtual RFLP patterns. Cucumber-associated Turkish '*Ca*. P. trifolii' isolate shared a virtual RFLP similarity coefficient 0.98 with the stains of 16SrVI: clover proliferation group (subgroup VI-A), while the '*Ca*. P. solani' shared a similarity coefficient 1.00 with phytoplasmas of 16SrXII: Stolbur group (subgroup XII-A) (Figure 4).

A 2-base insertion has detected in the 16S rRNA gene of Van-trifolii isolate which distinguished from other world isolates of 16SrVI clover proliferation group (subgroup VI-A) phytoplasma. As shown in Figure 5 in circle, the R16F2n/R16R2 sequence of Van-trifolii isolate had specific insertions of 2 bp at different positions of the rRNA gene (positions 789 and 940) that differed from the same nucleotide positions in '*Ca*. P. trifolii' isolates.

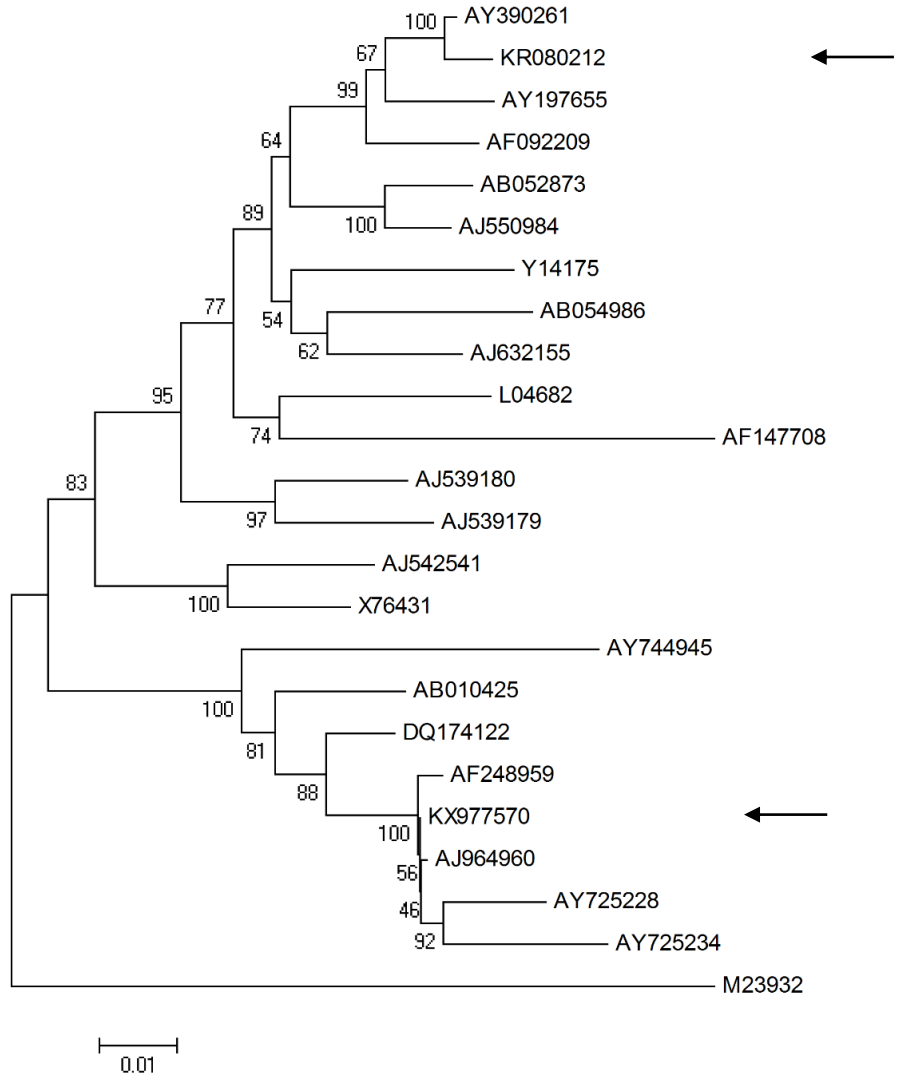
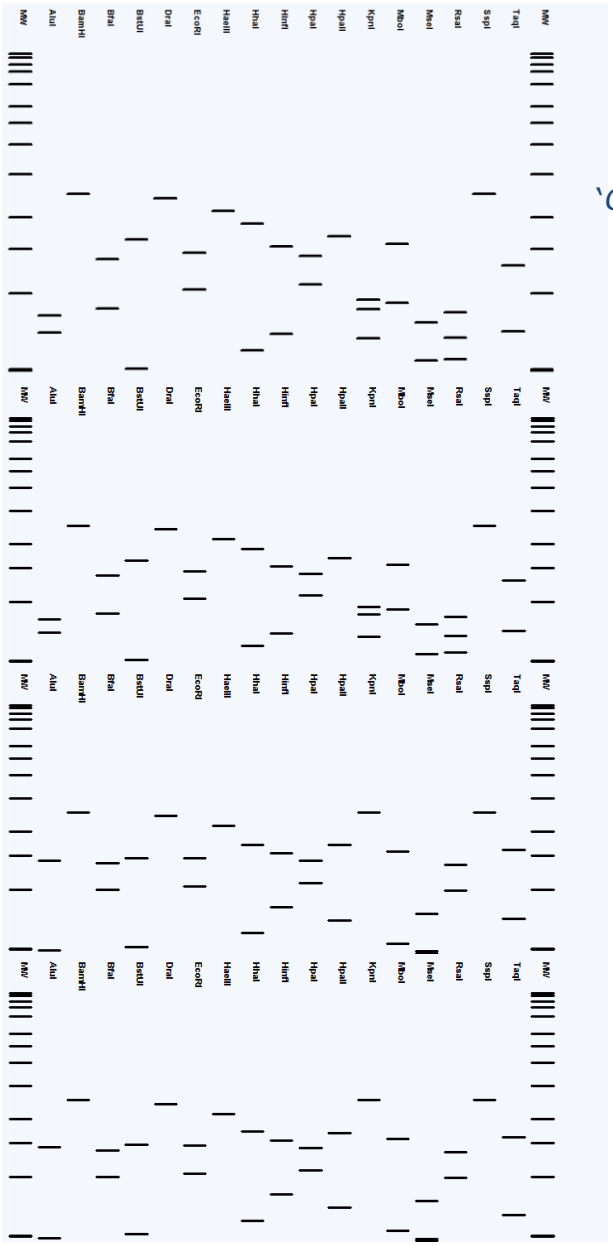


Figure 3. Phylogenetic tree constructed with neighbor joining algorithm of 16Sr-DNA sequences of '*Ca*. P. solani' and '*Ca*. P. trifolii' and *A. laidlawii* was taken as the outgroup to root the tree. Bootstrap values for 1000 replicates are shown on branches. The arrows indicate the position of Turkish isolates. Sequences for '*Ca*. Phytoplasma'species were retrieved from NCBI Genbank. '*Ca*. P. trifolii; AY390261 (Hiruki and Wang 2004), '*Ca*. P. trifolii'; KR080212 (this publication), '*Ca*. P. ulmi'; AY197655 (Lee et al. 2004), '*Ca*. P. fraxini'; AF092209 (Griffiths et al. 1999), '*Ca*. P. oryzae; AB052873 (Jung et al. 2003), '*Ca*. P. cynodontis'; AJ550984 (Marcone et al. 2004b), Phytoplasma sp. strain LDN; Y14175 (Tymon et al. 1998), '*Ca*. P. castaneae'; AB054986 (Jung et al. 2002), '*Ca*. P. pini'; AJ632155 (Schneider et al. 2005), Western X-disease phytoplasma L04682; (GenBank submission, 1999), '*Ca*. P. brasiliense'; AF147708 (Montano et al. 2001), Sugarcane phytoplasma D3T2; AJ539180 (GenBank submission, 2003), Sugarcane phytoplasma D3T1; AJ539179 (GenBank submission, 2003), '*Ca*. P. mali'; AJ542541 (Seemüller and Schneider 2004), '*Ca*. P. rhamni'; X76431 (Marcone et al. 2004a), Derbid phytoplasma; AY744945 (GenBank submission, 2004), '*Ca*. P. japonicum'; AB010425 (Sawayanagi et al. 1999), '*Ca*. P. americanum'; DQ174122, (Lee et al. 2006), '*Ca*. P. solani' STOL; AF248959, '*Ca*. Phytyoplasma solani'; KX977570 (this publication), '*Ca*. P. solani'; AJ964960 (Firrao et al. 2005), '*Ca*. P. graminis'; AY725228 (Arocha et al. 2005), '*Ca*. P. caricae'; AY725234 (Arocha et al. 2005), Acholeplasma laidlawii; M23932.



'*Ca*. Phytoplasma solani' "STOL"
16SrXII-A (AF248959)

'*Ca*. Phytoplasma solani' "Van-solani"
16SrXII-A (AF248959)

'*Ca.* Phytoplasma trifolii'16SrVI subgroup VI-A AY390261

'*Ca.* Phytoplasma trifolii'16SrVI, subgroup VI-A "Van-trifolii"
KR080212

Figure 4. Virtual R16F2n/R16R2 RFLP patterns of representative strains of 16SrVI clover proliferation group (subgroup VI-A) and 16SrXII Stolbur group (subgroup XII-A) comparing with the Turkish isolates of the same groups, MW: 1 kb Promega DNA ladder.

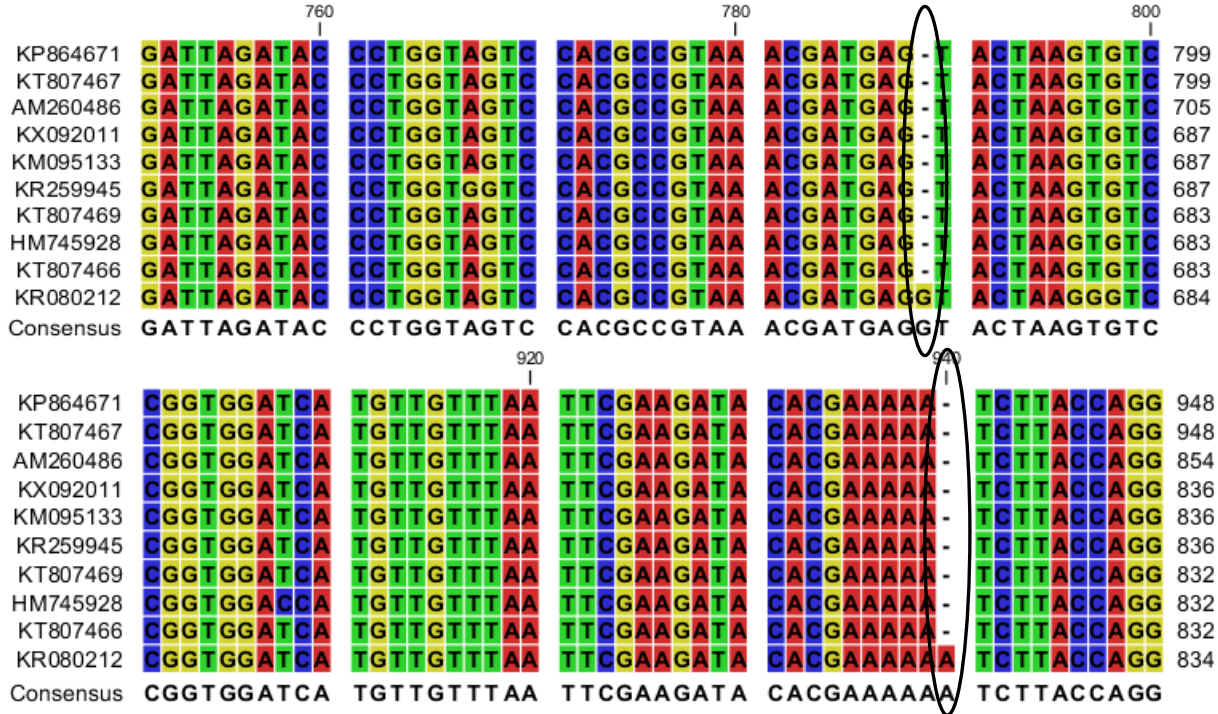


Figure 5. Partial sequence alignment of '*Ca*. P. trifolii' Van-trifolii isolate (KR0802212) with available world isolates classified in group 16SrVI clover proliferation (subgroup VI-A) phytoplasma. Base insertions differing from those of world isolates are circled. The isolates used in alignment are: KP864671 (Russia), KT807467 (Iran), AM260486 (Lebanon), KX092011 (Mexico), KM095133 (Mexico), KR259945 (Mexico), KT807466 (Iran), KR080212 (Turkey, Van-trifolii)

Discussion

A PCR band of approx. 1.25 kb was obtained from severely symptomatic cucumber plant samples with the same size as the positive control when the 16S rDNA was amplified by Nested-PCR using universal primers R16F2/R2. No PCR products were obtained from asymptomatic field cucumber plants.

In 1998, Lee et al. were used 17 restriction enzymes to differentiate various phytoplasmas by their distinct RFLP patterns of 16S rDNA. The *in silico* virtual RFLP profiles of Van-solani isolate were identical to that of reference strain '*Ca*. Phytoplasma solani' "STOL"16SrXII-A (AF248959) which was reported as Stolbur phytoplasmas (SeemullerSeemüller et al. 1994). Likewise, virtual RFLP profiles of Van-trifolii isolate were identical to that of reference strain '*Ca*. Phytoplasma trifolii' 16SrVI-A clover proliferation phytoplasma (AY390261). Wei et al. (2007) showed that the virtual 16S rRNA gene RFLP patterns of previously classified strains matched perfectly with the RFLP patterns on real gels. Their results suggest that the virtual RFLP analysis could be used as a reliable and convenient alternative to conventional RFLP analysis.

In cucumber, phytoplasmas have been reported to be associated to disease, belonging to different groups (Montano et al. 2006; Montano et al. 2007). The phytoplasmas of 16SrVI clover proliferation (subgroup VI-A) have been reported to infect many plant species in the world (Khasa et al. 2016; Zibadoost et al. 2016; Choueiri et al. 2007). However, there is no previous report of subgroup 16SrVI-A isolates infecting cucumber in Turkey.

Over the past 15 years, the RFLP profiling of 16S rRNA gene fragments has served as a primary means for identification, classification and differentiation of known phytoplasmas (Zhao et al. 2009). The sequence information is still very useful tool in studying phytoplasma features (Contaldo et al. 2012; Lou et al. 2013). PCR assays using 16S rRNA gene primers and subsequent molecular cloning and bidirectional sequencing have successfully identified the presence of subgroup 16SrVI-A and 16SrXII-A pathogens as phytoplasma and detected it in infected cucumber. Therefore, these molecular tools can provide a robust and sensitive diagnosis to detect and predict the occurrence of phytoplasma epidemics (Wu et al. 2010).

In the present study, we have provided evidence that '*Ca*. P. solani' and 'Ca. P. trifolii' are found to be associated with severe diseases in cucumber in Van Province of Turkey. Two different Turkish phytoplasma isolates (Van-solani and Van trifolii) were characterized. Further studies are required to investigate the insect vectors responsible for the phytoplasma transmission in the region and potential weed host range of the agents.

Acknowledgement

This study was supported by the grant (Project No: FHD-2016-5205) from Research Fund of Van Yuzuncu Yil University.

References

Ahrens U, Seemüller E (1992). Detection of DNA of plant pathogenic mycoplasma like organisms by a polymerase chain reaction that amplifies a sequence of the 16S RNA gene. Phytopathology. 82: 828-832.

Alp Ş, Usta M, Sipahioğlu HM, Güller A (2016). First report of "*Candidatus* Phytoplasma solani" on a new host marigold (*Tagetes erecta* L.). Turk. J. Agric. For. 40: 311-318.

Al-Saady NA, Khan AJ, Calari A, Al-Subhi AM, Bertaccini A (2008). "*Candidatus* Phytoplasma omanense", associated with witches-broom of *Cassia italica* (Mill.) Spreng. in Oman. Int. J. Syst. Evol. Microbiol. 58: 461-466.

Arocha Y, Lopez M, Pinol B, Fernandez M, Picornell B, Almeida R, Palenzuela I, Wilson MR, Jones P (2005). '*Candidatus* phytoplasma graminis' and '*Candidatus* phytoplasma caricae', two novel phytoplasmas associated with diseases of sugarcane, weeds and papaya in Cuba. Int. J. Syst. Evol. Microbiol. 55: 2451-2463.

Başpınar H, Korkmaz S, Önelge N, Çınar A, Uygun N, Kersting U (1993). Studies on Citrus Stubborn Disease pathogen and Sesame Phyllody MLO and their associated leafhopper vectors in sesame. J. Turk. Phytopathol. 1: 1-8.

Bertaccini A, Duduk B, Paltrinieri S, Contaldo N (2014). Phytoplasmas and Phytoplasma Diseases: A Severe Threat to Agriculture. Am. J. Plant Sci. 5: 1763-1788.

Choueiri E, Salar P, Jreijiri Fouad, Zammar SE, Masaad R, Abdul-Nour H, Bove´ JM, Danet JL, Foissac X (2007). Occurrence and distribution of '*Candidatus* Phytoplasma trifolii' associated with diseases of solanaceous crops in Lebanon. Eur. J. Plant. Pathol. 118: 411-416.

Contaldo N, Bertaccini A, Paltrinieri A, Windsor HM, Windsor GD (2012). Axenic culture of plant pathogenic phytoplasmas. Phytopathol. Mediterr. 51: 607-617.

Çağlar BK, Elbeaino T (2013). A novel phytoplasma associated with witches' broom disease of *Ligustrum ovalifolium* in Turkey. J. Plant Pathol. 137: 113-117.

Çağlar BK, Elbeaino T, Küsek M, Pehlivan D, Fidan H, Portakaldalı M (2010). Stolbur Phytoplasma Infections in Potato and Tomato Plants from Different Locations in Turkey. J. Turk. Phytopath. 39: 1-3.

Davis RI, Schneider B, Gibb KS (1997). Detection and differentiation of phytoplasma in Australia. Aust. J. Agric. Res. 48: 535-544.

Duduk B, Bertaccini A (2011). Phytoplasma classification: Taxonomy based on 16S ribosomal gene, is it enough. Phytopathogenic Mollicutes. 1 (1): 3-13.

Firrao G, Gibb K, Streten C (2005). Short taxonomic guide to the genus '*Candidatus* Phytoplasma'. J. Plant Pathol. 87: 249-263.

Gazel M, Çağlayan K, Başpınar H, Mejia JF, Paltrinieri S., Bertaccini A, Contaldo N (2016). Detection and Identification of Phytoplasmas in Pomegranate Trees with Yellows Symptoms. J. Phytopathol. 164: 136-140.

Griffiths HM, Sinclair WA, Smart CD, Davis RE (1999). The phytoplasma associated with ash yellows and lilac witches'- broom: '*Candidatus* Phytoplasma fraxini'. Int. J. Syst. Bacteriol. 49: 1605-1614.

Gundersen DE, Lee IM (1996). Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. Phytopathol. Mediterr. 35: 144-151.

Hiruki C, Wang KR (2004). Clover proliferation phytoplasma: '*Candidatus* Phytoplasma trifolii'. Int. J. Syst. Evol. Microbiol. 54: 1349-1353.

Hogenhout SA, Oshima K, Ammar El-D, Kakizawa S, Kingdom HN, Namba S (2008). Phytoplasmas: bacteria that manipulate plants and insects. Mol. Plant Pathol. 9 (4): 403-423.

IRPCM Phytoplasma/Spiroplasma Working Team–Phytoplasma Taxonomy Group (2004). '*Candidatus* Phytoplasma', a taxon for the wall-less, non-helical prokaryotes that colonize plant phloem and insects. Int. J. Syst. Evol. Microbiol. 54: 1243-1255.

Jung HY, Sawayanagi T, Kakizawa S, Nishigawa H, Miyata SI, Oshima K, Ugaki M, Lee JT, Hibi T, Namba S (2002). '*Candidatus* Phytoplasma castaneae', a novel phytoplasma taxon associated with chestnut witches' broom disease. Int. J. Syst. Evol. Microbiol. 52: 1543-1549.

Jung HY, Sawayanagi T, Wongkaew P, Kakizawa S, Nishigawa H, Wei W, Oshima K, Miyata S, Ugaki M, Hibi T (2003). '*Candidatus* Phytoplasma oryzae', a novel phytoplasma taxon associated with rice yellow dwarf disease. Int. J. Syst. Evol. Microbiol. 53: 1925-1929.

Khan AJ, Botti S, Subhi AMA, Zaidi MA, Altosaar I, Alma A, Bertaccini A (2003). Molecular Characterization of the 16S RNA gene of phytoplasma detected in two leafhopper species associated with alfalfa plants infected with witches' broom in Oman. Phytopathol. Mediterr. 42 (3): 257-267.

Khasa E, Gopala Taloh A, Prabha T, Madhupriyal, Rao GP (2016). Molecular characterization of phytoplasmas of 'Clover proliferation' group associated with three ornamental plant species in India. 3 Biotech. 6 (2): 237.

Lee IM, Bottner KD, Secor G, Rivera-Varas V (2006). '*Candidatus* Phytoplasma americanum', a phytoplasma associated with a potato purple top wilt disease complex. Int. J. Syst. Evol. Microbiol. 56: 1593-1597.

Lee IM, Davis RE (1988). Detection and investigation of genetic relatedness among aster yellows and other mycoplasma-like organism by using cloned DNA and RNA probes. Mol. Plant-Microbe Interact. 1: 303-310.

Lee IM, Gundersen-Rindal DE, Davis RE, Bartoszyk IM (1998). Revised classification scheme of phytoplasmas based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences. Int. J. Syst. Bacteriol. 48: 1153-1169.

Lee IM, Hammond RW, Davis RE, Gundersen DE (1993). Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasma-like organisms. Phytopathology. 83: 834-842.

Lee IM, Martini M, Macone C, Zhu SF (2004). Classification of phytoplasma strains in the elm yellows group (16SrV) and proposal of '*Candidatus* Phytoplasma ulmi' for the phytoplasma associated with elm yellows. Int. J. Syst. Evol. Microbiol. 54: 337-347.

Lou B, Bai X, Bail Y, Deng C, RoyChowdhury M, Chen C, Song Y (2013). Detection and Molecular Characterization of a 16SrII-A phytoplasma in Grapefruit (*Citrus paradisi*) with Huanglongbing-like Symptoms in China. J. Phytopath. 162: 387-395.

Marcone C, Gibb KS, Streten C, Schneider B (2004a). '*Candidatus* Phytoplasma spartii', '*Candidatus* Phytoplasma rhamni' and '*Candidatus* Phytoplasma allocasuarinae', respectively associated with spartium witches'-broom, buckthorn witches'-broom and allocasuarina yellows diseases. Int. J. Syst. Evol. Microbiol. 54: 1025-1029.

Marcone C, Schneider B, Seemüller E (2004b). '*Candidatus* Phytoplasma cynodontis', the phytoplasma associated with Bermuda grass white leaf disease. Int. J. Syst. Evol. Microbiol. 54: 1077-1082.

Montano HG, Davis RE, Dally EL, Hogenhout S, Pimentel JP, Brioso PST (2001). '*Candidatus* Phytoplasma brasiliense', a new phytoplasma taxon associated with hibiscus witches' broom disease. Int. J. Syst. Evol. Microbiol. 51: 1109-1118.Montano HG, Brioso PST, Pimentel JP, Figueiredo DV, Cunha JO (2006). *Cucurbita moschata*, new phytoplasma host in Brazil. J. Plant Pathol. 88 (2): 225-229.

Montano HG, Paulo STB, Roberta CP, Joao PP (2007). *Sicana odorifera* (Cucurbitaceae) a new phyto-plasma host. Bull. Insectology. 60: 287-288.

Murray RGE, Stackebrandt E (1995). Taxonomic Note: Implementation of the Provisional Status Candidatus for Incompletely Described Procaryotes. Int. J. Syst. Bacteriol. 45 (1): 186-187.

Murray RGE, Schleifer KH (1994). Taxonomic notes: a proposal for recording the properties of putative taxa of procaryotes. Int. J. Syst. Bacteriol. 44 (1): 174-176.

Namba S, Kato S, Iwanami S, Oyaizu H, Shiozawa H, Tsuchizaki T (1993). Detection and differentiation of plant-pathogenic mycoplasma-like organisms using Polymerase Chain Reaction. Phytopathology. 83: 786-791.

Özdemir N, Saygılı H (2012). Batı Anadolu Bölgesi Domates Üretim Alanlarında Görülen Stolbur Hastalığının Tohumla Taşınıp Taşınmadığı İle İlgili Bir Araştırma. U. Ü. Ziraat Fakültesi Dergisi. 26 (1): 63-72.

Özdemir N, Saygılı H, Sahin F, Karsavuran Y, Bayrak OF, Oral B (2009). Host range and genetic characterization of a phytoplasma causing tomato stolbur disease in Turkey. Proc. IInd Intl. Symposium on tomato diseases. Acta Hort. 808: 255-261.

Sawayanagi T, Horikoshi N, Kanehira T, Shinohara M, Bertaccini A, Cousin MT, Hiruki C, Namba S (1999). '*Candidatus* Phytoplasma japonicum', a new phytoplasma taxon associated with Japanese Hydrangea phyllody. Int. J. Syst. Bacteriol. 49: 1275-1285.

Schneider B, Torres E, Martin MP, Schroder M, Behnke HD, Seemuller E (2005). '*Candidatus* Phytoplasma pini', a novel taxon from *Pinus silvestris* and *Pinus halepensis*. Int. J. Syst. Evol. Microbiol. 55: 303-307.

Seemüller E, Schneider B, Maurer R, Ahrens U, Daire X, Kison H, Lorenz KH, Firrao G, Avinent L, Sears BB (1994). Phylogenetic classification of phytopathogenic mollicutes by sequence analysis of 16S ribosomal DNA. Int. J. Syst. Bacteriol. 44 (3): 440-446.

Seemüller E, Schneider B (2004). Taxonomic description of '*Candidatus* Phytoplasma mali' sp. nov., '*Candidatus* Phytoplasma pyri' sp. nov. and '*Candidatus* Phytoplasma prunorum' sp. nov., the causal agents of apple proliferation, pear decline and European stone fruit yellows, respectively. Int. J. Syst. Evol. Microbiol. 54: 1217-1226.

Sertkaya G, Osler R, Musetti R, Ermacora P, Martini M (2004). Detection of phytoplasmas in Rubus spp. by microscopy and molecular techniques in Turkey. Acta Hort. 656: 181-186.

Sertkaya G, Martini M, Ermacora P, Musetti R, Osler R (2005). Detection and characterization of phytoplasmas in diseased stone fruits and pear by PCR-RFLP analysis in Turkey. Phytoparasitica. 33: 380-390.

Sertkaya G, Martini M, Musetti R, Osler R (2007). Detection and molecular characterization of phytoplasmas infecting sesame and solanaceous crops in Turkey. Bull. Insec.. 60 (2): 141-142.

Sertkaya G, Martini M, Osler R (2008). First report of *Candidatus* phytoplasma mali in Turkey. J. Plant Pathol. 90 (1): 143-149.

Tamura K, Dudley J, Nei M, Kumar S (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24: 1596-1599.

Tymon AM, Jones P, Harrison NA (1998). Phylogenetic relationships of coconut phytoplasmas and the development of specific oligonucleotide PCR primers. Ann. Appl. Biol. 132: 437-452.

Ulubaş Serçe Ç, Gazel M, Çaglayan K, Baş M, Son L (2006). Phytoplasma diseases of fruit trees in germplasm and commercial orchards in Turkey. J. Plant Pathol. 88 (2): 179-185.

Wei W, Davis RE, Lee IM, Zhao Y (2007). Computer simulated RFLP analysis of 16S rRNA genes: identification of ten new phytoplasma groups. Int. J. Syst. Evol. Microbiol. 57: 1855-1867.

Wu Y, Hao X, Li Z, Gu P, An F, Xiang J, Wang H, Luo Z, Liu J, Xiang Y (2010). Identification of the phytoplasma associated with wheat blue dwarf disease in China. Plant Dis. 94: 977-985.

Zhao Y, Wei W, Lee IM, Shao J, Suo X, Davis RE (2009). Construction of an interactive online phytoplasma classification tool, iPhyClassifier, and its application in analysis of the peach X-disease phytoplasma group (16SrIII). Int. J. Syst. Evol. Microbiol. 59: 2582-2593.

Zibadoost S, Rastgou M, Asghari S, Tazehkand SA (2016). Detection and molecular identification of '*Candidatus* phytoplasma trifoli' infecting some cultivated crops and vegetables in West Azarbaijan province. Australas. Plant Dis. Notes. 11: 3.