Stolbur Phytoplasma Infections in Potato and Tomato Plants from Different Locations in Turkey

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ABSTRACT

During August 2012, a survey and identification of phytoplasmas associated with diseased potato (*Solanum tuberosum* L.) and tomato (*Solanum lycopersycum* L.) plants were conducted in four regions, in Turkey. Potato samples with reddish or purplish discoloration and rolling of leaves symptoms were gathered from "Kayseri and Sivas" provinces, whilst tomato samples were collected form plants exhibiting floral abnormalities, sepal hypertrophy, virescence and phyllody symptoms, from Kahramanmaraş and Adana provinces. All symptomatic plants of both species reacted positively when assayed by direct polymerase chain reactions (PCR) using universal primer pair R16F1/R16R0 and nested PCR using R16F2n/R16R2 primers. Phytoplasmas were detected in 32 symptomatic plants, out of 40 samples collected. However, no PCR amplicon products were obtained from the asymptomatic ones (8 plants). BLAST sequence analysis of the 16SrDNA amplicons (1250 bp) showed that the phytoplasma found in potato and tomato samples resembled "*Candidatus phytoplama solani*" (16SrXII-A ribosomal subgroup member) and shared with this last 99.8% sequence identity. Similar PCR and sequence results were obtained from *Cicadula inornata* (*Cicadellidae*), insects collected from affected tomato plants in surveyed fields, when were assayed by PCR and 16SrDNA-sequenced. The RFLP profile of the 1250 bp PCR fragments, restricted with 7 different endonucleases (*Eco*RI, *Taq*I, *HhaI*, *Alu*I, *Mse*I, *Rsa*I and *Hp*aII) usually used for phytoplasma subgroups differentiation, showed identical patterns to "*Candidatus phytoplasma solani*". RFLP results were in harmony with the phylogenetic tree constructed with the sequences obtained, which grouped in one cluster all stolbur phytoplasma from Turkey and those of 16SrXII-A ribosomal subgroup members. To our knowledge, all phytoplasma diseases were detected from potatoes and tomatoes in Kayseri, Sivas, Adana and Kahramanmaraş provinces are same phytoplasma. Further investigations are needed to determine whether *Cicadula inornata* insect, trapped from affected plants is a potential vector responsible for the transmission of this phytoplasma in that area, where tomatoes are grown in Turkey.

Key words: 16S rRNA, PCR, RFLP, sequencing and phylogenetic analysis

INTRODUCTION

Phytoplasma of the stolbur group (16SrXII) are phloem limited, insect-transmitted pathogens and they infect a great number of plants species (Ember *et al*., 2011; Lee *et al*. 2000), and depending on the affected species this phytoplasma induce various systemic symptoms ranging from yellowing, shoot proliferation, witches'-broom growth to phyllody and virescence. In the last ten years, increasing incidence of stolbur phytoplasma was registered in different crops (grapevine, maize, sugar beet, potato, tomato, vegetable crops), suggesting its' progressive spread. In the vegetable crops, severe yield losses caused by stolbur phytoplasma have been recorded in solanaceous crops (tomato, potato, pepper) and celery (Carraro *et al*., 2008; Navràtil *et al*., 2009; Fialova *et al*., 2009, Ember *et al*., 2011). Stolbur phytoplasma also has a wide host range that includes weeds from the families *Asteraceae* (*Taraxacum officinale*, *Cirsium arvense*), *Convolvulaceae* (*Convolvulus arvensis*) and *Urticaceae* (*Urtica dioica*), which can serve as pathogen reservoirs (Berger *et al*. 2009; Navràtil *et al*. 2009; Langer and Maixner 2004).

Potatoes and tomatoes are considered among the most important crops in Turkey, as their total productions reach high levels (4,648,081 and 11.003.433 tons, respectively) (Anonymous, 2011). The presence of stolbur phytoplasma disease in potato and tomato fields of Turkey has been recorded (Özdemir *et al*., 2009), however not much is known regarding the molecular information of its' genome.

Accordingly, the first aim of this study is to investigate the etiology of diseased potato and tomato plants in four provinces in Turkey, Kayseri, Sivas, Kahramanmaraş, and Adana. Surveyed fields showed plants with leaf yellowing and rolling, reddish and purplish discoloration and presence of aerial tubers, whislt tomato plants exhibited flower malformations, phyllody and big bud, all symtpoms suspected to be of phytoplasmal origin.

The second aim is to characterize at the molecula level the identified pathogen reponsible of the encountered diseases, for which the results are hereafter reported.

MATERIALS AND METHODS

Plant Material and Insects Sampling

During August 2012, two lots of 20 samples each were collected from four locations, Kayseri/Sivas and Kahramanmaraş/Adana, dedicated to potato and tomato plantations, respectively. Samples consisted of leaves collected from plants showing typical symptoms of phytoplasma (Fig. 1 and 2) and from plants with apparent symptoms. The survey has also interested the collection of *Cicadula inornata* (*Cicadellidae*) insects that were found nourishing on infected plants. Insects were trapped with a D-Vac apparatus in tomato plots at Kahramanmaraş (Elbistan) province. Samples gathered from potatoes fields at Kayseri province were denoted "PoSKa2" and "PoSKa4", from Sivas province as "PoSSi1" and "PoSSi5", while samples gathered from tomatoes fields from Adana provinces were denoted "ToSAd1" and "ToSAd2". Tomato samples and *Cicadula inornata* from tomato in Kahramanmaraş (Elbistan) were denoted "ToSEL1" and "ToSCi" respectively. All samples originated from plants and insects were subjected to molecular assays.

DNA Extraction and Polymerase Chain Reaction Amplification (PCR)

DNA was extracted from fresh leaves of symptomatic and asymptomatic tomato and potato plants as described by Ahrens and Seemüller (1992), with some modifications. Tissue samples (1 g) were homogenized in 4 ml of CTAB buffer (2% w/v cetyltrimethylammonium bromide, 1.4 M NaCl, 0.2% 2-β-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, 2% polyvinylpyrrolydone, pH 8.0) and 1.5 ml aliquots of the extract were incubated at 65°C for 30 min. DNA was further purified by phenol and chloroform-isoamyl alcohol (24:1) extraction and afterward precipitated. Eluted DNA template was used for direct PCR amplification. DNA was also extracted from cixiids according to Doyle and Doyle (1990).



Figure 1. (a) Potato plants infected with stolbur phytoplasma and showing aerial tubers with severe deformation symptoms. (b) Tomato plants infected with stolbur phytoplasma and showing sepal hypertrophy symptoms.

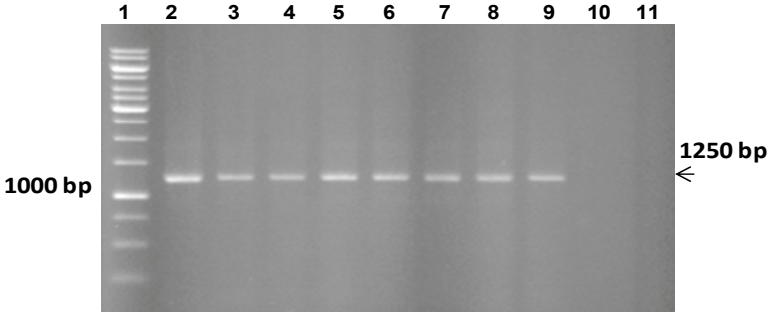


Figure 2. Electropherogram showing 16SrDNA nested-PCR products amplified with R16F2n/R16R2 from potato and tomato phytoplasma-affected plants. Lane 1: 1 kb DNA marker; lane 2: PoSKa2; lane 3: PoSKa4; lane 4: PoSSi1; lane 5: PoSSi5; lane 6: ToSEL1; lane 7: ToSCi; lane 8: ToSAd1; lane 9: ToSAd2; lane 10: healthy potato plant; lane 11: healthy tomato plant.

The universal phytoplasma primer pair R16F1/R16R0 (5'-AAGACGAGGATAACAGTTGG-3'/5'-GGATACCTTGTTACGACTTAACCCC-3') (Lee *et al*., 1994) was used in one step PCR for amplifying a 1.8 kbp fragment of ribosomal operon consisting of the 16SrRNA gene, the 16S-23S intergenic spacer region (SR) and a portion of the 5' region of 23SrRNA gene. A 1:100 dilution of the single step PCR product was used as template for

the nested PCR round, utilizing the primer pair R16F2n/R16R2 (5'-ACGACTGCTAAGACTGG-3'/5'-TGACGGGCGGTGTGTACAAACCCCG-3'), which amplify an internal DNA fragment of 1,250 bp from the 16SrRNA gene (Gunderson and Lee, 1996).

For first step PCR, amplification was performed in 50 reaction mixtures, each containing 100 ng of extracted DNA, 1.25 µl dNTPs (10 mM), 1 µl forward and reverse primers (10 pmol), 10 µl of 5X Crimson Taq reaction buffer, 3 µl $MgCl_2$ (25 mM) and 0.25 µl Crimson Taq DNA polymerase (5U/µl) (BioLabs, USA). PCR was conducted in a Techne TC 4000 apparatus using the following parameters: 35 cycles of 1 min at 94 °C, 2 min at 50 °C and 3 min at 72 °C. PCR conditions for the second round (nested PCR) were the same, except for the annealing temperature that was at 58 °C. An extension cycle consisting of 10 min at 72 °C was used for both PCRs. 10 µl of PCR products primed with R16F2n/R16R2 were electrophoresed in 1% agarose gel in 1xTBE buffer (67 mM Tris-HCl, 22 mM boric acid, 10 mM EDTA, pH 0.8) together with 1 kb DNA marker (Fermentas, Milan, Italy), stained with ethidium bromide and photographed on a UV transilluminator.

Restriction Fragment Length Polymorphism (RFLP) Analyses

Restriction fragment length polymorphism (RFLP) analysis was performed using 100 ng of purified R16F2n/R16R2-primed nested-PCR amplicons products obtained from potato and tomato samples and reference stolbur strain. First RFLP was performed using *Eco*RI to distinguish between R16F2n/R16R2 PCR amplicon product of phytoplasma and chloroplast DNA of plants (Nejat et. al 2009).

Amplicons were digested separately with 2 µl each of following restriction enzymes: *Taq*I at 65°C and *Mse*I, *Alu*I, *Hpa*II, *Hha*I, *Rsa*I at 37°C, according to manufacturer's instruction (Fermentas, Milan, Italy). Fragments patterns were compared after electrophoresis on a 5% polyacrylamide gel followed by ethidium bromide staining, and photographed under UV at 312 nm using a transilluminator.

Cloning, Sequencing and Phylogenetic Analysis

The R16F2n/R16R2 primed-16S rDNA PCR products obtained from stolbur phytoplasma infected plants were excised from agarose gel, washed and eluted by centrifugation through siliconized glass wool, as described by Gromadka (1995). The eluted DNAs were sequenced from both directions using M13 forward and reverse sequencing-primers. DNA fragments were subjected to automated sequencing (ABI 3130xl Genetic Analyzer, Applied Bio. REFGEN Gen Araştırmaları ve Biyoteknoloji Ltd. Şti., Ankara, Turkey).

Computer-assisted analysis of nucleotide sequences was assembled using the Strider 1.1 program (Marck, 1988). 16S-23S rDNA sequences of stolbur phytoplasma isolates with similar reference phytoplasmas were separately aligned using Clustal X 1.81 (Thompson *et al*., 1997). Phylogenetic tree was constructed using the NJ plot and Boostrap analysis with 1000 replicates using the NEIGHBOR methods of the PHYLIP package (Felsenstein, 1989).

RESULTS

Detection of Phytoplasma and Sequence Analysis

The presence of phytoplasma was detected in 32, out of 40 tested, symptomatic potato and tomato plants (Fig. 1) resulting with an amplification of 1,250 bp DNA fragments using nested-PCR (Fig. 3). No PCR positive reactions were obtained from the eight asymptomatic plants tested. BLAST sequence analyses conducted on 20 different recombinant DNA clones, originated from 10 different infected plants of each species, showed that the 16SrDNA fragments amplified from potatoes and tomatoes plants share the highest identity (99.8%) with stolbur phytoplasma and in particular with the "*Candidatus phytoplasma solani*", 16SrXII-A subgroup member, from Serbia (accession number: JQ730750) and Romania (accession number: HQ108388). All 16SrDNA phytoplasmal sequences of stolbur found in potato and tomato in Turkey were identical (100% homology) when compared between them.

RFLP Analysis

The presence of a single *Eco*RI restriction site in the 16F2n/R16R2- primed PCR products (1250 bp) generated a RFLP pattern made of two DNA fragments (750 bp and 500 bp), thus ascertaining the phytoplasmal nature of the nested-PCR amplicon (Figure 3). Performing separate digestions of PCR products with different endonucleases (*Eco*RI, *Taq*I, *Hh*aI, *Alu*I, *Mse*I, *Rsa*I and *Hp*aII), all samples showed identical restriction profiles (Figure 3), thus indicating that a single phytoplasma infection has occurred in all affected plants. The RFLP profiles from stolbur phytoplasma isolates of Turkey and of that found in *Cicadula inornata* insects, were all similar to that of the reference strain, *"Candidatus phytoplasma solani"*, showing that there is no genetic variability within the Turkish isolates.

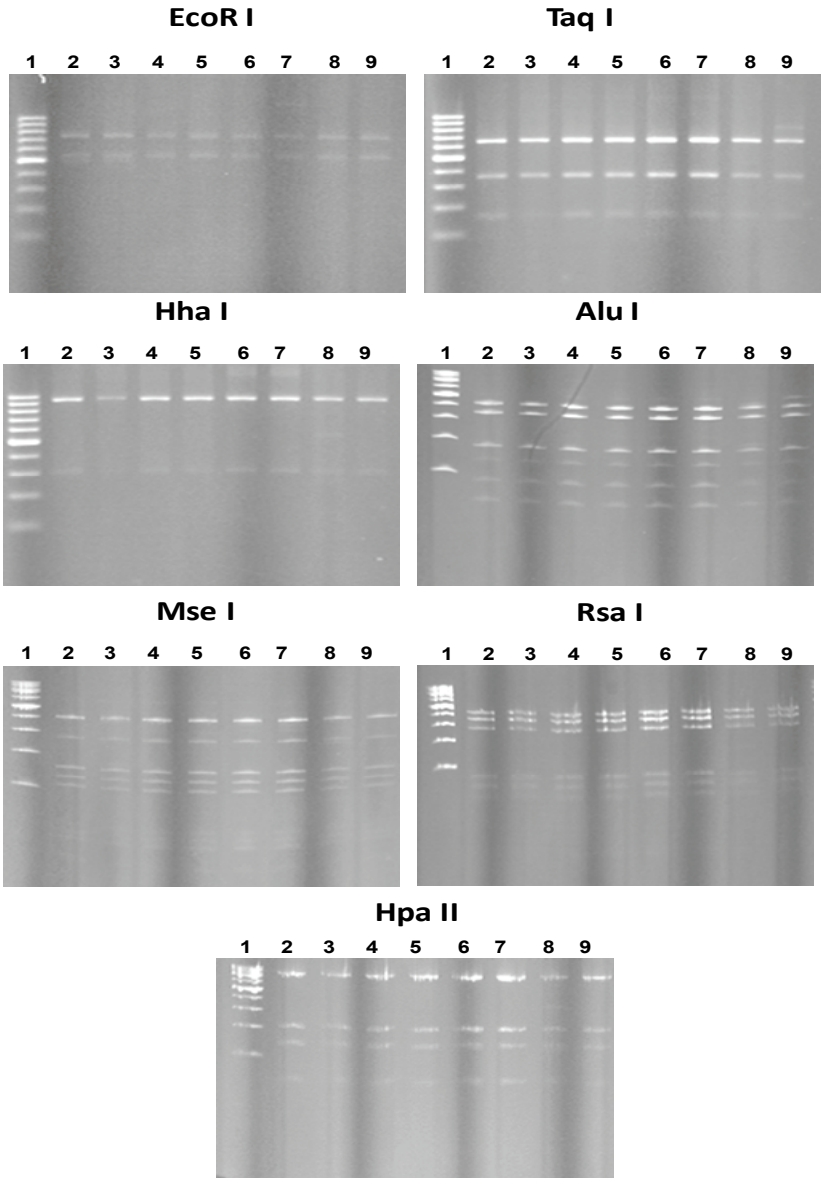


Figure 3. Electropherogram showing Restriction Fragment Length Polymorphisms of 16SrDNA, amplified by nested-PCR from diseased potato and tomato plants, using seven restriction enzymes (indicated above gels). Lane 1: 1 kb DNA marker; lane 2: PoSKa2; lane 3: PoSKa4; lane 4: PoSSi1; lane 5: PoSSi5; lane 6: ToSEL1; lane 7: ToSCi; lane 8: ToSAd1; lane 9: Tomato plant infected with "*Candidatus phytoplasma solani*" (16SrXII-A) used as reference control.

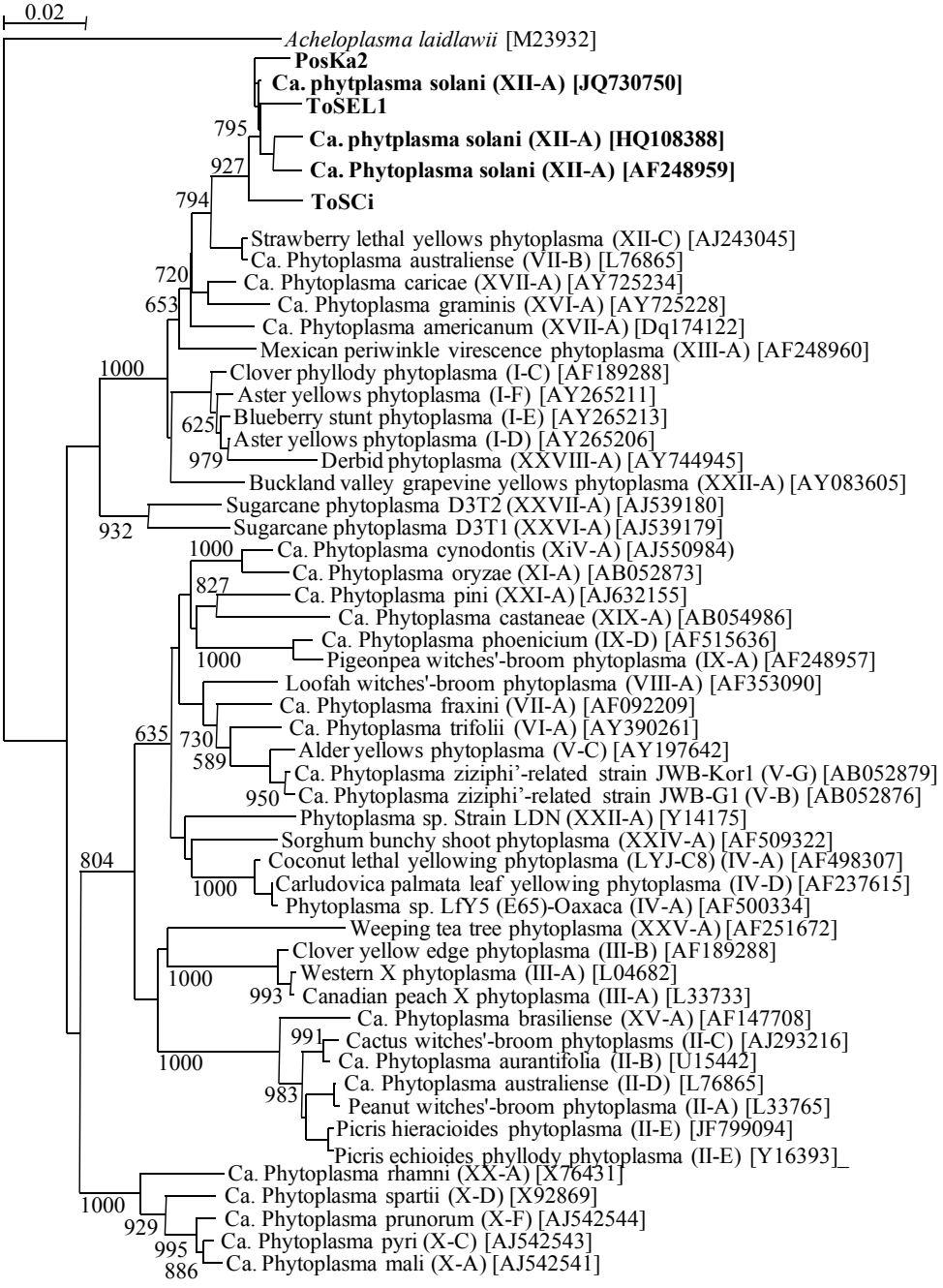


Figure 4. Dendrogram, constructed by the neighbour-joining method, showing the phylogenetic relationship of stolbur phytoplasma from potato (Poska2), tomato (ToSEL1) and cixiid (ToSCi) of Turkey (in bold) and those present in database, based on 16S rRNA gene sequences. Groups and subgroups of phytoplasmas are reported between brackets ( ). GenBank accession numbers for sequences are reported between two braces [ ]. The reliability of the analysis was subjected to a bootstrap test with 1000 repeats. Bar, 0.01 nucleotide substitutions per site. *Acheloplasma laidlawii* was used as an outgroup species to root the tree.

Phylogenetic Tree

The phylogenetic tree constructed with 16SrDNA sequences of the stolbur phytoplasma of potato and tomato, together with members of 16SrXII-related subgroups, confirmed the RFLP results, hence placed the stolbur phytoplasma from Turkey in one subclade together with 16SrXII subgroups members (Figure 4).

DISCUSSION

In recent years, emerging phytoplasma diseases have increasingly become important in Turkey, due to the increment of plant material exchanging between agricultural districts and to the moderate weather that favors the multiplication of these pathogens and their insects-vectors. The molecular investigation carried out on samples collected from diseased potato and tomato plants, and originated from four different regions particularly dedicated to their production, showed the presence of a high level of phytoplasma infections; specifically with stolbur phytoplasma "*Candidtus phytoplasma solani*", 16SrXII-A ribosomal subgroup member. The incidence of this phytoplasma in the surveyed field was somehow significant (80%), considering that 32 samples were PCR-positive out of 40 tested. The "*Candidtus phytoplasma solani*", was previously reported in solanaceous crops in Turkey, however with a lower incidence (Sertkaya *et al*., 2007). Accordingly, quarantine measures should be taken in order to prevent the further expansion of this pathogen where potatoes and tomatoes are grown in the country.

The molecular analysis conducted in this study showed that there is no notable sequence variation in the stolbur phytoplama found in both species. This result was also confirmed with the RFLP, sequencing and phylogeny analyses.

An interesting outcome of this study was the identification of stolbur phytoplasma in *Cicadula inornata* insects (*Cicadellidae*); however, further experiments are needed to ascertain whether this phytoplasma is transmitted in nature by this novel insect.

ÖZET

TÜRKIYE'NIN DEĞIŞIK YÖRELERINDEN ALINAN PATATES VE DOMATES BITKILERINDE GÖRÜLEN STOLBUR PHYTOPLASMA ENFEKSIYONLARI

Türkiye'nin dört farklı ilinde 2012 Ağustos ayı boyunca yapılan bir survey süresince patates (*Solanum tuberosum* L.) ve domates (*Solanum lycopersycum* L.) bitkilerinde fitoplasma kaynaklı hastalığın tanılanması ile ilgili bir çalışma yürütülmüştür. Kayseri ve Sivas illerinden kızarıklık, morarma gibi renk değişikliği ve yaprak kıvrılması simptomu gösteren patates bitki örnekleri, Kahramanmaraş ve Adana illerinden çiçek anormallikleri, çanak yaprak irileşmesi ve şişmesi simptomu gösteren domates bitki örnekleri toplanmıştır. Toplanan örnekler Polimeraz Zincir Reaksiyonu (PCR) yöntemi ile R16F1/R16R0 (universal) ve R16F2n/R16R2 (nested) primerler kullanılarak testlendiğinde, simptom gösteren bütün bitkiler pozitif reaksiyon vermiştir. Araştırmaya dahil edilen toplam 40 bitki örneğinden 32 simptomlu bitkide fitoplazma saptanırken, 8 simptomsuz bitkide fitoplazma saptanmamıştır. Domates ve pataterslerde saptanan fitoplazmanın 16S rDNA'i üzerinden yapılan PCR sonucunda elde edilen PCR ürününün (1250 bp) baz dizilimi, "*Candidatus phytoplama solani*" (16SrXII-A ribosomal altgurub) ile %99.8 oranında benzerlik göstermiştir. Domates üretim alanlarından toplanan *Cicadula inornata* (Cicadellidae)'nın bünyesindeki fitoplazmanın, 16S rDNA'i üzerinde yapılan PCR ve genetik analizlerden de aynı sonuç elde edilmiştir. İzolatlar arasında genetik farklılıkların olup olmadığını kontrol etmek amacıyla PCR sonucunda elde edilen 1250 bp PCR ürünü *Eco*RI *Taq*I, *HhaI*, *Alu*I, *Mse*I, *Rsa*I *Hp*aII enzimleri kullanılarak RFLP çalışmasına dahil edilmiştir. RFLP sonuçlarında *Candidatus phytoplasma solani* ile aynı sonucu vermiştir. RFLP sonuçlarının ve genetik dizilimle elde edilmiş filogenetik ağacın uyum içerinde olduğu ve Türkiye'deki araştırmaya konu olan stolburun 16SrXII-A ribosomal altgruba ait olduğu ortaya konulmuştur. Çalışmada Kayseri, Sivas, Adana ve Kahramanmaraş illerindeki patates ve domateslerden saptanan fitoplazmaların tamamı benzer olarak bulunmuştur. Türkiye'deki üretim alanlarında *Cicadula inornata*'nın fitoplazma vektörü olup olmadığı konusunda da daha fazla araştırma gerekmektedir.

Anahtar Kelimeler: 16S rRNA, PCR, RFLP, genetik dizilim ve filogenetik analiz

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