Yuzuncu Yil University
Journal of Agricultural Sciences
(Yüzüncü Yıl Üniversitesi Tarım Bilimleri Dergisi)
https://dergipark.org.tr/en/pub/yyutbd

ISSN: 1308-7576 e-ISSN: 1308-7584

Research Article

First Report of '*Candidatus* Phytoplasma australasia' Strain Related to Witches'-Broom of Tomato in Türkiye

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Article Info

Received: 15.11.2022
Accepted: 19.01.2023
Online published: 15.03.2023
DOI:10.29133/yyutbd.1205091

Keywords

16S rRNA,
Molecular phylogeny,
Phloem restricted bacteria,
RFLP,
Solanum lycopersicum

Abstract: Phytoplasmas are dangerous bacteria severely infecting agricultural production worldwide. In the present study, the identification of phytoplasmas infecting tomato plants showing symptoms such as small leaves, flower abnormalities, stunting, witches' broom, and reddening was performed. Five plants, two symptomatic and three asymptomatic, were tested to verify phytoplasma infection. Total DNA isolated from 5 leaf samples was used as a template for PCR reactions. The phytoplasma agents were confirmed in the two symptomatic samples. BLASTn search of 16S rRNA of two sequences shared identity similarity of 99.84% with '*Candidatus* Phytoplasma australasia'. Computer-simulated virtual RFLP profiles show that the 16S rRNA sequences is identical to the reference pattern of the 16SrII-D subgroup, with a similarity coefficient of 1.00. Based on BLAST, virtual RFLP, and phylogenetic dendrogram, the identified phytoplasma strains are enclosed in the 16SrII-D subgroup. This is the first report of tomato witches' broom disease related to 16SrII-D subgroup phytoplasma strains in the Antalya province of Türkiye.

To Cite: Usta, M, Güller, A, Sipahioğlu, H M, 2022. First Report of '*Candidatus* Phytoplasma australasia' Strain Related to Witches'-Broom of Tomato in Türkiye. *Yuzuncu Yil University Journal of Agricultural Sciences*, 33(1): 54-63.
DOI: https://doi.org/10.29133/yyutbd.1205091

1. Introduction

Tomatoes are one of the most popular vegetables in the world, with high economic value and health benefits. At a country level, China is the largest producer in 2019 and accounted for 62.7 million tons in production and Türkiye ranks third with 12.8 million tons. In Türkiye, tomato production is made mainly in Antalya, Bursa, Manisa, and Izmir provinces (CIA, 2017). Tomato (*Solanum lycopersicum* L.) is a basic host of many pathogenic microorganisms, including fungi, viruses, bacteria, viroids, and phytoplasmas. Among them, phytoplasmas were first evaluated as viruses or virus-like diseases, then named mycoplasma-like organisms (MLO) in 1967, and finally called 'Phytoplasma' in 1992. Up to now, these pathogens are categorized into 33 taxonomic groups that rely on the 16Sr ribosomal RNA gene, each comprising a different number of subgroup members (Bertaccini and Lee, 2018). Phytoplasmas, belonging to the '*Candidatus* Phytoplasma' genus, are the smallest living microorganisms and are devoid of the cell wall. Some basic genes are missing from the small genome with a low G+C content (Oshima et al., 2013).

Their infection in over 700 plants involving annual and perennial plants, fruit and forest tree, and ornamental plants is a huge constraint on agricultural production, which leads to a significant loss of quality and quantity (Zibadoost et al., 2016; Venkataravanappa et al., 2017). As it damages the vegetative and generative organs, product losses because of infection in cultivated plants such as cucumber, tomato, and eggplant can reach up to 100% in distinct ecological zones (Rao and Kumar 2017; Kumari et al., 2019). Microscopic techniques have been used mainly in the diagnosis of phytoplasmas, they do not allow for adequate identification (Namba 2019). Currently, nucleic acid-based techniques (16S rRNA, SecA, SecY, Tuf gene) are a reliable tool for identifying this pathogen and ensuring the group/subgroup separation to which it belongs (Venkataravanappa et al., 2017; Usta et al., 2021). A total of seven main 'Ca. Phytoplasma' species have so far been identified from tomato: '*Ca*. P. lycopersici' and '*Ca*. *P. asteris*' in Bolivia and Poland (Arocha et al., 2007; Krawczyk et al., 2010), '*Ca*. P. aurantifolia' and '*Ca*. P. australasia' in China and India (Singh et al., 2012; Dong et al., 2013), '*Ca*. P. pruni' in Brazil (Amaral-Mello et al., 2006), '*Ca*. P. trifolii' and '*Ca*. P. solani' in Türkiye (Usta et al., 2018), '*Ca*. P. ulmi' in central Italy (Del Serreno et al., 2001).

Phytoplasmas are related to metabolic and phenotypic alterations in their hosts and affect the fecundity of the plant. The associated with all phytoplasmas symptoms infecting tomatoes are close to each other. The characteristic ones appearing are yellowing, lateral shoots, little young leaves, fruit abnormalities, reddening, over-longed calyx, purple leaves, and witches' broom (Xu et al., 2013).

Greenhouse vegetable cropping has been one of the important livelihoods in Kaş (Antalya, Türkiye). In this district, tomato plants are grown in heated greenhouses in an area devoted to protected-culture vegetable production (Gül and Özenç, 2020). The symptoms of tomato-phytoplasma disease have been identified in different regions of Turkey (Güller and Usta, 2020; Çağlar and Şimşek, 2022). However, there is no scientific report related to the Antalya province. The objective of this study is to identify the phytoplasma presence and identity of diseased tomato plants from greenhouse-grown tomatoes in the Antalya province of Turkey.

2. Material and Methods

2.1. Source of tomato plants

In the spring of 2020, 5 tomato samples (2 symptomatic and 3 symptomless) were collected from three greenhouses in the Kaş district (Antalya, Turkey). All plant samples were transported to the virology laboratory of Van Yuzuncu Yil University for molecular analyses and stored at -20 °C prior to molecular tests.

2.2. DNA Extraction and molecular amplification (PCR)

Total DNA extraction was done from 0.5 g of tomato leaf tissue using a commercial plant DNA extraction kit (Thermo Fisher, USA) according to manufacturer's guidance by grounding through a ball-bearing homogenizer. The purified DNA was subjected to PCR to provide amplification of the 16S rRNA gene by direct PCR and nested PCR steps. PCR-targeting universal primers (R16mF2/R16mR1 for d-PCR and R16F2n/R16R2 for n-PCR) amplifying a segment of 1.8 kb and 1.25 kb, for both steps were adopted as specified by Lee et al., (1993) and Gundersen and Lee (1996), respectively. The reaction parameters and temperature cycles were applied as described by Usta et al., (2018). To ensure the reliability of the PCR assays, DNA of '*Ca*. P. solani' (KX977570) was used as a positive control, as well as, DNA from symptom-free plants and DNA-free mix were used as negative controls. The d-PCR yields were diluted 1:30 ratio and employed as templates for re-amplification in n-PCR. Following n-PCR, amplified DNAs of 20 µl and DNA ladder (3.0 kb) as size standard were electrophoretically fractionated in agarose gel stained with EtBr (Ethidium Bromide) buffered in 1×TAE (Tris Acetic EDTA) and visualized under UV light with a digital imaging device (SYNGENE).

2.3. Cloning, sequence identity, and identification of phytoplasma

Amplified products were gel-purified using a kit (Thermo Fisher, USA) from the supplier. Standard cloning protocol was used to transfer the eluted product (1µl) into a bacterial cloning vector (pGEM T- Easy) (Promega, Madison, USA).

Recombinant plasmids were electroporated into *E. coli* JM109 competent cells. White recombinant colonies consisting of insert DNA were selected from solid medium containing ampicillin (1%) and cultured in a liquid LB medium containing the same amount of antibiotics. To detect the relevant gene sequence of the pathogen, recombinant plasmids were purified from the cell suspension and sequenced by next generation sequencing (NGS) (Sentebiolab/Ankara/Turkey). The sequence identities were analyzed using BLASTn queries in the GenBank database.

2.4. Consensus tree and in silico RFLP Analysis

*i*PhyClassifier program was used to calculate the similarity coefficient and to determine the group and subgroup of sequences of R16F2n/R16R2-primed n-PCR yields. For further characterizations, *in silico* digestion was also conducted using seventeen key digestion 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 by pDRAW32program (Zhao *et al.,* 2013). The restriction profiles of '*Ca*. P. australasia' Antalya strains were compared with that of the reference strain (Access no: Y10096). A phylogenetic dendrogram was constructed from Antalya strains along with a set of 27 available 16S rRNA sequences archived into NCBI. *Acholeplasma palmae* (Accession no: L33734) was selected as the outsource to ensure a valid rooting. The consensus tree was constructed by the maximum likelihood algorithm using CLC Main workbench software. Tree branches were bootstrapped with 1000 replicates to assess the accuracy of the concluded clades and sub-clades.

3. Results

3.1. Visual assessment

Symptoms were usually conspicuous in infected tomatoes with floral and leaf symptoms. The most commonly observed symptoms on greenhouse-grown tomato plants ranged from mild to severe floral sterility and abnormalities, no leaves on the branch tips, adventitious shoot, hypertrophied calyx, witches' broom, curled and purplish leaves, and yellowing and small deformed young leaves (Figure 1).

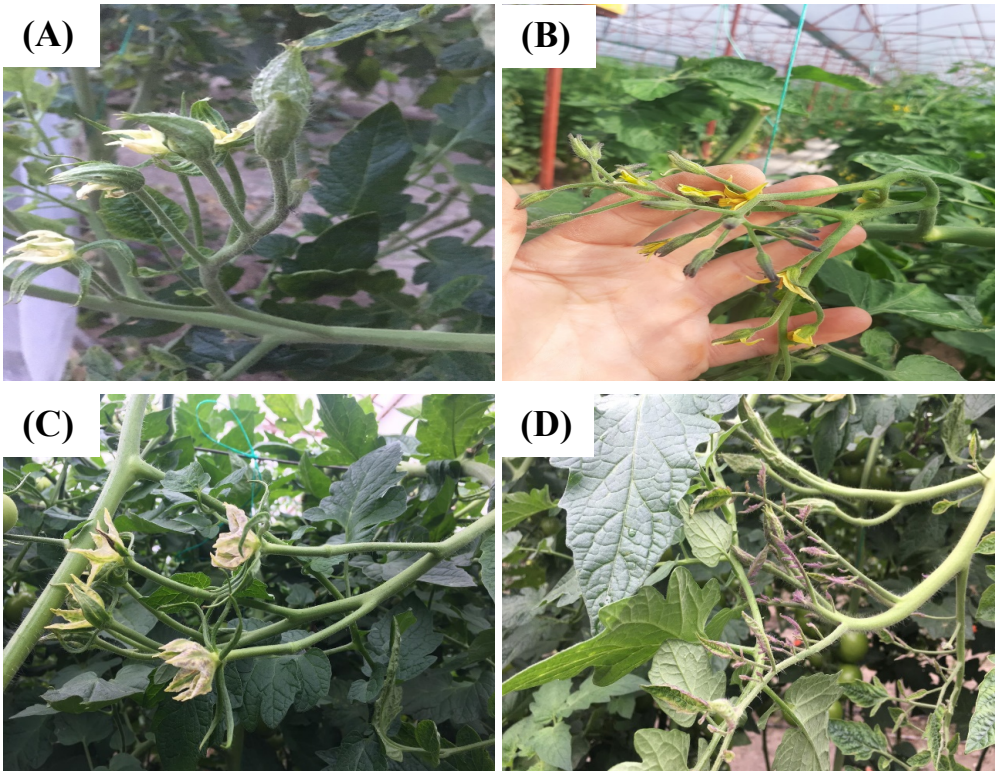


Figure 1. **A-** Heavily phytoplasma infected tomato plants growing in different greenhouses in Kaş district. Big bud, **B, C-** floral sterility and abnormalities, **D-** purplish small sized leaves due to '*Ca*. P. australasia' infections.

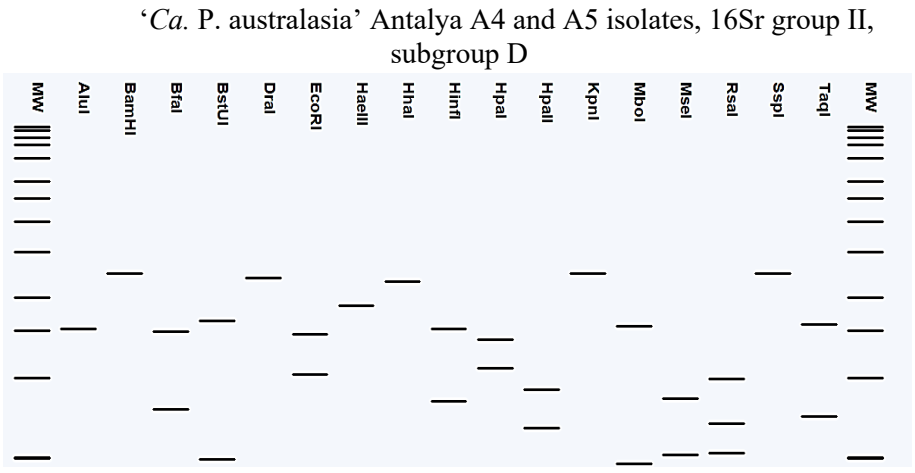
3.2. Detection of phytoplasma, sequence identity and similarity coefficients

In direct PCR tests, the universal R16mF2/R16mR1 primer pair generated DNA fragments of 1.8 kb from positive control and two symptomatic tomato samples (data not shown), but not from symptomless samples and negative control. The occurrence of phytoplasmas was identified in 2 out of 5 analyzed, resulting in an expected size of 1.25 kb nested-PCR amplicons primed with R16F2n/R16R2, corresponding to the 16S rRNA gene of the pathogen. Two cloned obtained 16S rRNA sequences displayed 99.92% nucleotide identity with that of reference strains '*Ca*. P. australasia' (GenBank accession no: Y10096).

A sequence BLAST search of F2nR2 fragments proved that the two tomato strains from this study have a 99.84% identity with many phytoplasma members 16SrII '*Ca*. P. australasia' strains (Accession no. OM416010, OM416008, OL306323, OK644503, OM415996, i.e.) and '*Ca.* P. aurantifolia' (Accession no. OK625583, MZ348527). Based on *i*Phyclassifier software, the OM513906 and OM616883 sequences, identified in this study, were '*Ca*. P. australasia' (16Sr II-D subgroup, Peanut witches' broom (PnWB)), with a 1.00 similarity coefficient.

3.3. Virtual RFLP and phylogenetic analysis

Virtual RFLP studies also confirmed the groups to which tomato-related phytoplasmas belong. The cut profile of the two '*Ca*. P. australasia' sequences were identical with (1.00 similarity coefficient) the reference strain of '*Ca*. P. australasia' (Accession no.Y10096), demonstrating no genetic diversity (Fig. 2). The present finding pointed out a minor difference in the 16S rRNA gene sequence of 16Sr group II, subgroup D compared to that of the 16Sr group II, subgroup B reference isolate(Access no. U15442). This assignment was also corroborated by the clustering assays with other '*Ca*. Phytoplasma' strains in primarily 16SrII and other groups retrieved from the NCBI (Figure 2).



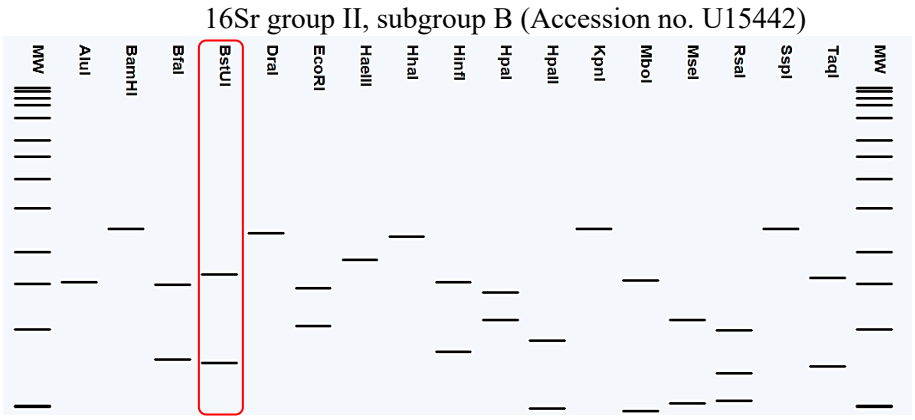


Figure 2. Virtual restriction endonuclease digestion patterns of tomato-associated '*Ca*. P. australasia' A4 (Accession no.OM513906) and A5 (Accession no.OM616883) isolates. No differences have been identified in restriction patterns of two isolates (A4 and A5) and the reference strain of 16SrII-D subgroup (Accession no. Y10096). However, a slight difference has been identified in electrophoretic mobility between the members of 16SrII-D subgroup and the reference strain in 16SrII-B subgroup (Accession no. U15442) when digested with *Bst*UI (shown in box). M: 100 bp DNA size standard.

The phylogenetic tree of the aligned 16S rRNA sequences showed that tomato phytoplasmas from Kaş (Antalya) clustered with '*Ca*. P. australasia' strains belonging to the Peanut witches'-broom phytoplasma group, more specifically with the 16SrII-D subgroup (Figure 3).

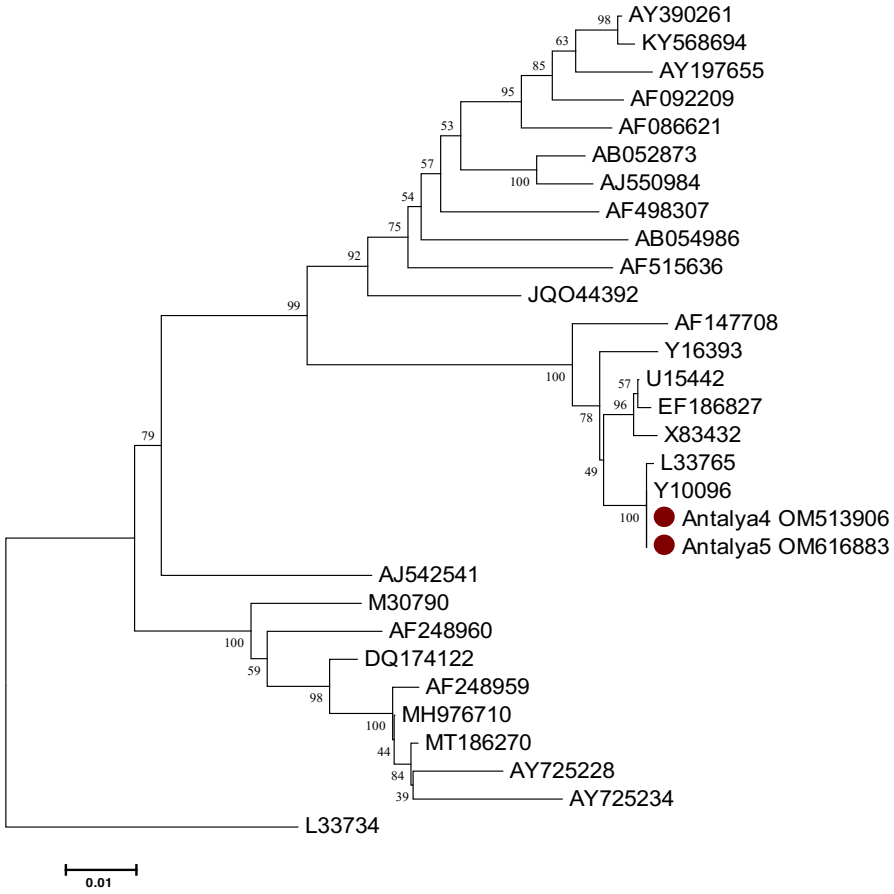


Figure 3. Phylogenetic dendrogram of 16Sr RNA gene sequences of 'Ca. P. australasia' A4 and A5 strains constructed with maximum likelihood algorithm using the reference strains and other 'Ca. Phytoplasma' species and isolates. L33734 (*Acholeplasma palmae* 16S rRNA gene) was added as outgroup. The bootstrap score of 1000 replicates as percent are shown on branches.

4. Discussion

Phytoplasma-infected tomato plants from various 16S ribosomal groups have been described in various countries (Contaldo et al., 2021; Davoodi et al., 2019; Khalil et al., 2019; Oksal, 2020). In the present study, the '*Ca*. P. australasia' (16SrII-D subgroup) was identified in phytoplasma-symptomatic tomato plants in Turkey, which is the first record nationally (Fig 2). In Turkey, phytoplasmas belong to eight 16Sr groups which are I, IX, and X (Canik et al., 2011, 2019), II (Ayvaci et al., 2020), V (Sertkaya et al., 2004), VI and XII (Usta and Güller, 2018), and XIV (Çağlar et al., 2013) have been identified in different hosts, including ornamental plants, orchards, weeds, and cultured crops.

'*Ca*. P. australasia' (16SrII-D subgroup) is associated with a multitude of plant diseases. The new data is important for the locality in terms of agent/disease transmission. In Turkey, Ayvaci et al., (2020), Özdemir and Cagirgan (2015), and Ozdemir et al., (2014) described this pathogen in alfalfa (*Medicago sativa*), jute (*Corchorus olitorius*) plants exhibiting symptoms such as little leaves, leaf yellowing, and witches' broom and in Orosius orientalis, respectively. The molecular characterization of this strain in tomato affirmed the 'Ca. P. australasia' identity and represented a new epidemiological host about this agent in Turkey. Because of the prevalence of tomato cultivation, this pathogen has a potential to cause epidemics in other crop plants when the insect vector(s) and alternative hosts are available. Similar morphological symptoms triggered by the same phytoplasma in tomato plants were described in India (Singh et al., 2012), in Egypt and Iraq (Omar and Foissac, 2012; Alkuwaiti et al., 2017), in Iran (Salehi et al., 2016).

The 16SrII-D ribosomal subgroup of phytoplasmas has a diverse spectrum of hosts with geographical prevalence, including *Petunia violacea*, *Calendula officinalis*, alfalfa, squash, pomegranate, and parsley in Iran (Salehi et al., 2014; Esmailzadeh Hosseini et al., 2015, 2018; Hemmati et al., 2019); *Cycas revoluta* and *Phoenix dactylifera* in Oman (Hemmati et al., 2020a; 2020b); periwinkle, onion, and *Opuntia abjecta* in Egypt (El-Sisi et al., 2017); sweet potato, pale purple coneflower, *Echinacea pallida* and papaya in Australia (Pearce and Scott 2017; White et al., 1998), tomato, eggplant and mallow in Iraq (Alkuwaiti et al., 2017); *Daucus carota* and sesame in India (Venkataravanappa, 2017); eggplant in China (Li et al., 2019); sesame, faba bean, and chickpea in Pakistan and Sudan (Akhtar et al., 2009; Alfaro-Fernández et al., 2012).

Based on this, a rapid and accurate diagnosis of phytoplasma infection in economically important plants is important for the surveillance and management of the disease. In the past, the identification and taxonomy of phytoplasmas were based on the sensitivity of the test plants, their induced symptoms, and their relationship with insect hosts (Shiomi and Sugiura 1984; Chiykowski 1991). Today, RFLP methods of the conserved 16S rRNA marker gene sequence and DNA hybridization method have been a basic tool adopted by most researchers for precise detection and differentiation of phytoplasma infections (Zelyut et al., 2022). In this study, we employed the 16S rRNA gene region, which is widely used in phytoplasma taxonomy. Although this sequence is valuable for revealing interspecies taxonomic levels, a multilocus analysis should be performed using the rp, secY, secA, cpn60, and tuf gene locus to distinguish closely related phytoplasma strains in the same 16Sr groups and gain advantages over single gene analysis (Davis et al., 2013; Dumonceaux et al., 2014; Serçe and Yılmaz 2019; Valiunas et al., 2019).

Management of phytoplasma diseases is extremely troublesome. Many methods have been tried and no single effective method has been developed. Chemicals such as chloramphenicol, tetracycline and salicylic acid, antimicrobial compounds, and developing resistant cultivars are sufficiently ineffective because of both costly and no long-term protective ways, although they provide symptom remission (Bertaccini, 2021; Upadhyay, 2016). Therefore, the most plausible approach strategically is that prophylactic methods restricting the expansion dissemination of these agents may be more usable and sustainable. Control of weeds or insects is important for inoculum formation, its transmission and disease development (Cagirgan et al., 2014). Further studies are needed to determine the distribution, inoculum sources and insect vectors of '*Ca*. P. australasia' in these areas where protected edible crops are grown. In this way, it can be ensured that the agent is brought under control before it reaches the threshold of economic threat.

Conclusion

In the present study, the etiology and epidemiology of phytoplasma bacteria, one of the dangerous pathogens of agriculture and production, are evaluated. In Turkey, the new host of the tomato plants, '*Ca*. Phytoplasma australasia' has been detected using PCR-based RFLP approaches and phylogenetic analyzes based on the 16s rRNA gene of the relevant pathogen. This is the first report of '*Ca*. P. australasia' (16SrII group/D subgroup) associated with Witches'-Broom of tomato in Turkey. The outputs of this study will shed light on the epidemiology of phytoplasma diseases in Turkey.

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