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**ARAŞTIRMA MAKALESİ** **RESEARCH ARTICLE**

# '*Candidatus* Phytoplasma solani' (Subgroup 16SrXII-A) Associated with *Nicotiana tabacum* Leaf Abnormality in Turkey

Türkiye'de *Nicotiana tabacum* Yaprak Anomalileri ile İlişkili '*Candidatus* Phytoplasma solani' (Alt grup 16SrXII-A)

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## Abstract

Tobacco (*Nicotiana tabacum*) is among the agricultural products with the highest added value in Turkey. Although frequently associated with its negative effects on human health, it also provides important contributions to the Turkish economy with the employment it creates in rural areas and continues to be a strategic product. Many postgraduate theses and studies related to the sociological and economic importance of the production of this plant, which is of great importance for our country, have been carried out. However, there are very limited studies on plant diseases in tobacco production areas in Turkey. Phytoplasma is one of the important plant pathogens that cause yield loss in tobacco. Since available data on phytoplasma diseases on tobacco was very scarce worldwide, field surveys to collect samples showing phytoplasma infection-like symptoms such as yellowish color changes, leaf blisters, proliferation, dwarfism, and other physical abnormalities were carried out in Çanakkale and Balıkesir provinces of Turkey from June to August 2021. The presence of phytoplasmas in six samples was confirmed by 16S ribosomal DNA amplification by nested-PCR using universal phytoplasma primer sets, which also suggested the pathogen associated with the symptoms on tobacco. According to phylogenetic study and virtual-RFLP analysis using *Alu*I and *Mse*I endonuclease enzymes, the six Turkish tobacco phytoplasma strains all belong to group 16SrXII and have more than 99% nucleotide sequence identity with some members of '*Candidatus* Phytoplasma solani' of the taxonomic subgroup 'stolbur' (16SrXII-A). Genetic distances analysis indicated that group 16SrI was more closely related to 16SrXII than 16SrVI, in agreement with the groups clustering in the phylogenetic tree. Neutrality tests found that 16SrI and 16SrXII groups are experiencing expanding or bottleneck selections, probably due to new mutations in the 16S rRNA gene fragment. Meanwhile, 16SrVI populations are shown to be undergoing balancing selections, indicating that its isolates have evolved for a long time.

**Keywords:** Tobacco, phytoplasma, Genetic diversity, Phylogenetic, *In-silico* RFLP analysis

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## Öz

Tütün (*Nicotiana tabacum*) Türkiye'de katma değeri en yüksek tarımsal ürünler arasında yer almaktadır. İnsan sağlığına olan olumsuz etkileri ile sıklıkla gündeme gelmesinin yanı sıra, kırsal alanlarda oluşturduğu iş gücü ile de Türk ekonomisine önemli katkılar sunmakta ve stratejik bir ürün olma özelliğini devam ettirmektedir. Ülkemiz için büyük öneme sahip bu bitkinin üretiminin sosyolojik ve ekonomik önemi ile gerçekleştirilmiş çok sayıda lisansüstü tez ve çalışma mevcuttur. Ancak, Türkiye tütün üretim alanlarında bitki hastalıkları ile ilgili olarak gerçekleştirilmiş son derece sınırlı sayıda çalışma vardır. Tütünlerde verim kaybına neden olan önemli bitki patojenlerinden bir tanesi de fitoplazmadır. Tütün fitoplazma hastalıklarına ilişkin mevcut veriler dünya çapında çok az olduğundan dolayı, Çanakkale ve Balıkesir illerinde tütün ekim alanlarında sarımsı renk değişiklikleri, yaprak kabarcıkları, proliferasyon, cücelik ve diğer fiziksel anormallikler gibi fitoplazmaların neden olduğu semptomlar gösteren örneklerin toplanması için Haziran-Ağustos 2021 tarihleri arasında Türkiye'nin Çanakkale ve Balıkesir illerinde saha araştırmaları yapılmıştır. Altı örnekte fitoplazmaların varlığı, tütün semptomlarıyla ilişkili patojeni de öneren evrensel fitoplazma primer setleri kullanılarak nested-PCR ile 16S ribozomal DNA amplifikasyonu ile doğrulanmıştır. Filogenetik analizler ve *Alu*I ve *Mse*I endonükleaz enzimlerini kullanılarak uygulanan sanal-RFLP analizleri bu izolatların 16SrXII ''*Candidatus* Phytoplasma solani''(Stolbur) grubu ve 16SrXII-A altgrubu strainleri ile %99'dan fazla nükleotid dizi özdeşliğine sahip olduğunu göstermiştir. Genetik mesafe analizi, filogenetik ağaçta kümelenen gruplarla uyumlu olarak, grup 16SrI'nin 16SrXII ile 16SrVI'dan daha yakından ilişkili olduğunu göstermiştir. Tarafsızlık testleri ile, 16SrI ve 16SrXII gruplarının, muhtemelen 16S rRNA gen fragmentinde yeni mutasyonlar nedeniyle genişleyen veya darboğaz seçimlerinden geçtiğini göstermiştir. Ayrıca, 16SrVI popülasyonlarının uzun bir süredir evrimleştiğini gösteren dengeleme seçimlerinden geçtiği gösterilmiştir.

**Anahtar Kelimeler:** Tütün, Fitoplazma, Genetik çeşitlilik, Filogenetik, *İn-silico* RFLP analizi

## 1. Introduction

Tobacco (*Nicotiana tabacum*) produced in Turkey is in great demand due to its special quality and characteristics and is widely known as 'Turkish/Oriental Tobacco' in world markets (Wolf, 1949). Therefore, despite its adverse effects on human health, tobacco is still an important cultural plant in Turkey for the income it provides to farmers in the rural areas, and the generated tax revenue for the national economy (Yürekli et al., 2010). In Turkey, 90% of tobacco is grown in Western Anatolia and the rest in other regions (TSI, 2021).

Phytoplasmas are plant pathogenic prokaryotes belonging to the Mollicutes class due to their lack of a cell wall. They are obligate parasites which life cycle in infected plants was restricted only in the phloem (Marcone, 2014). Transmission between plants occurrs with the help of plant phloem-sap-feeding insects, such as leafhoppers, planthoppers, and psyllids. With very small genome of 680-1600 kilobases, the size of pleomorphic phytoplasmas varies from 200 to 800 nm (Bertaccini et al., 2014).

The pathogens are associated with hundreds of diseases, many are devastating ones; thus, reliable molecular tools including standard PCR and nested-PCR had been established for their proper identification. The molecular characterization of phytoplasmas was also used to discriminate them based on a particular 16S ribosomal gene sequence (16S rRNA) (about 1200 bp) into 33 groups which each have several subgroups (Bertaccini and Lee, 2018).

Viral diseases, other leading factors limiting tobacco production, has been relatively well studied globally (Stanković et al., 2011; Akinyemi et al., 2016), including in Turkey (Erdem, 2010; Çulal-Kılıç et al., 2017; Usta et al., 2020; Karanfil et al., 2020). On the other hand, there was no data related to the status of tobacco phytoplasma diseases, which are defined as closely related to virus diseases in terms of some symptomatological features, in Turkey. The description of natural infection of phytoplasma in other countries was also very scarce, although the physiological changes in tobacco inoculated with phytoplasma isolated from other plant species had been recorded (Lepka et al., 1999; Lherminier et al., 2003). Furthermore, data on the nucleotide sequence of tobacco phytoplasmas are limited in the NCBI GenBank.

In Turkey, the presence of phytoplasma in tobacco is highly suspected, as different groups of the pathogen have been previously identified in solanaceous and other crops (Çağlar et al., 2010; Usta et al., 2018; Yilmaz et al., 2019; Güller and Usta, 2020). Besides that, the causal agent of some viral-like tobacco diseases remains unresolved (Erdem, 2010; Çulal-Kılıç et al., 2017). On that account, field monitoring was carried out in Çanakkale and Balıkesir provinces, two important production centers of the Marmara and Aegean regions, to collect samples from plants with typical symptoms of phytoplasma disease. Samples were then molecularly analyzed to determine the infection rate and genetic diversity of phytoplasmas in the region. The outputs of this study could help to reduce the obvious worldwide knowledge gaps on phytoplasmas infecting tobacco.

## 2. Materials and Methods

### *2.1. Collection of phytoplasma sources*

Surveys were carried out in randomly selected tobacco fields representing different areas of Çanakkale and Balıkesir provinces which are located in the Southern Marmara Region in Turkey from June to August 2021. According to the size of the plant, whole or leaf samples were taken from plants symptomatic of phytoplasma infection. In addition, samples were taken from plants that did not show any symptoms. Samples were individually put inside a plastic bag containing silica gel then placed in a cooled icebox, and then brought to the laboratory to be stored at -20 °C until further tests.

### *2.2. Total nucleic acid isolation and Nested-PCR assays*

Total nucleic acid (TNA) was isolated from samples following a modified cetyltrimethylammonium bromide (CTAB) method developed by Li et al. (2008). The obtained DNA were kept at -20 °C until further application.

Nested-PCR (Nested-Polymerase Chain Reaction) modifications were applied in the amplifications of the targeted 16S rRNA gene region. TNAs concentration of each sample was spectrophotometrically measured using nano-drop (Thermo Sci., USA) and then adjusted by diluting with nuclease-free water. First PCR amplification was performed by mixing 25-30 ng/µl TNA, 0.4 µM R16mF2/R16mR2 universal primer pairs (Baric and Dalla-Via, 2004), 1.8 mM $MgCl_2$, 0.4 mM dNTP, 1.25 units Taq polymerase (Ampliqon, Denmark), 1X PCR buffer,

and nuclease-free water for a total volume of 25 µl per reaction tube.

Reactive products obtained from the first amplification were diluted with nuclease-free water at a ratio of 1:30 and then used as templates for the second reaction. The second reaction was carried out with 1 µl of the diluted reactive product, 0.4 µM R16F2n/R16R2 universal primers (Gundersen and Lee, 1996), and other components with the same mixing ratios of the first reaction. The well-characterized '*Candidatus* Phytoplasma trifolii (clover proliferation)' isolate BY11-2 (acc. no. MZ452938) and 'Carrot yellows phytoplasma (aster yellows)' isolate BD1-2 (acc. no. MZ452911) were also amplified as positive controls. The reaction products were run on a 1% agarose gel in 1X TAE buffer solution at 100 V for 1 hour. The agarose gel was then stained with ethidium bromide and visualized in a UV imaging device (Gene genius, UK). Successfully amplified DNA samples were submitted to BM Laboratory Systems, Ankara, Turkey for bidirectional sequencing using the Sanger method.

### *2.3. Phylogenetic analysis*

Nucleotide (nt) sequences of the 16S rRNA gene of the newly obtained isolates were subjected to BLAST analysis (blast.ncbi.nlm.nih.gov) to determine their identity rates with other phytoplasma isolates. 16Sr groups and subgroups with the highest nt identity to the new isolates and other groups and subgroups containing important strains were retrieved from the NCBI GenBank, then aligned with sequences of the new tobacco isolates using CLUSTAL W implemented in MEGA X v.10.2.4 (Kumar et al., 2018), and then trimmed according to the new isolate sequences length.

The best nt substitution model for the alignment was determined using the lowest BIC (Bayesian Information Criterion) scores in MEGA X v.10.2.4. The alignment was then subjected to a phylogenetic analysis using the Kimura-2-parameter model (Kimura, 1980) with Gamma distribution (shape parameter = 1) in the Maximum-Likelihood statistical method implemented in MEGA X v.10.2.4 (Kumar et al., 2018) to reveal the molecular evolutionary relation of the new and known phytoplasma isolates. Branches were supported by 1000 bootstrap replications. In addition, *Acholeplasma laidlawii* (acc. no. M23932), a non-cell wall bacterium genetically related to phytoplasmas, was used as an outgroup to increase the reliability of the main branches in the obtained dendrogram.

### *2.4.* In silico-*RFLP analysis*

To determine the 16Sr ribosomal groups and subgroups of new tobacco phytoplasmas, computer-simulated *in silico*-RFLP (restriction fragment length polymorphism) analysis was performed using *i*PhyClassifier software (Zhao et al., 2013). For this purpose, 16S rRNA-encoding gene sequences obtained were digested separately using 17commonly used restriction enzymes (*Rsa*I, *Ssp*I, *Taq*I, *Bfa*I, *BstU*I (*Tha*I), *Alu*I, *BamH*I, *Dra*I, *Hinf*I, *Hpa*I, *Eco*RI, *Hae*III, *Hha*I, *Sau3A*I (*Mbo*I), *Hpa*II, *Kpn*I, and *Mse*I). Comparisons were then made between the new isolates with reference isolates of the 16SrXII-A subgroup using *Alu*I and *Mse*I endonucleases. In addition, nt identity rates of the new isolates with reference isolates of the 16SrXII-A subgroup were also determined using the same software.

### *2.5. Genetic distances*

The mean evolutionary distance of the 16S rRNA gene of tested phytoplasma isolates within and between ribosomal groups 16SrI, 16SrXII, 16SrVI were calculated using the Kimura-2-parameter model (Kimura, 1980) in MEGA X v.10.2.4 (Kumar et al., 2018). Estimates of standard error (SE) were obtained by a bootstrap procedure (1000 replicates).

### *2.6. Genetic diversity and Neutrality analyses*

DnaSP v.6.12.03 software (Rozas et al., 2017) was used to measure the genetic diversity of the 16S rRNA gene of the new Turkish tobacco and other phytoplasma isolates. The three 'Neutrality tests': Fu and Li's F* and Fu and Li's D* (Fu and Li, 1993), and Tajima's D (Tajima, 1989) were carried out with a default window length of 100 sites and step size of 25 sites; without out-group using DnaSP v.6.12.03 (Rozas et al., 2017) to provide demographic data of the analyzed populations.

## 3. Results and Discussion

### *3.1. Symptomatology and Nested-PCR results*

A total of 61 samples, 32 from Çanakkale and 29 from Balıkesir, were collected during field surveys. The expected 1.2 kb fragments were obtained only in 1 out of 32 samples from Çanakkale and 5 out of 29 samples from Balıkesir. Symptomatologically, the positive tested samples were mostly plants with yellowish color changes in their leaves, leaf blisters, proliferation, dwarfism, and other physical abnormalities (*Figure 1*). The prevalence rate of phytoplasma was 9.84% in the surveyd regions, which indicates that phytoplasma infection is not common. This low incidence rate might be associated with plant resistance and also the limited number of vectors transmitting phytoplasma (Weintraub and Wilson, 2009). In addition, only a few vectors were observed since farmers, considering tobacco as a valuable commodity, routinely apply insecticides, including those used in planthopper and leafhopper controls, on their crops (Temel and Öztekin, 2020).

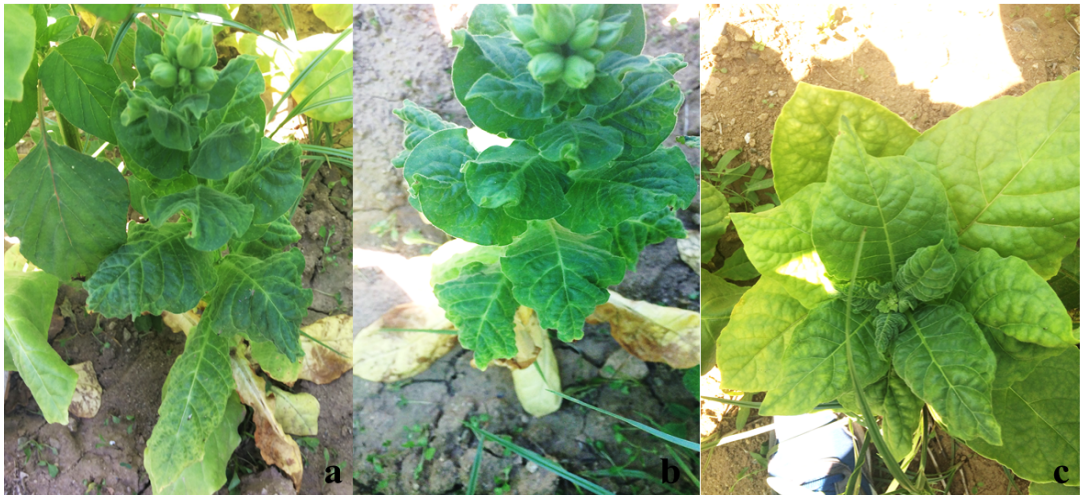


***Figure 1. Symptoms on tobacco plants infected with phytoplasma in Balıkesir province. (a) and (b). Conspicuous leaf blisters on lower leaves and plant dwarf; (c). Leaf yellowing, leaf proliferation, and plant dwarf***

### *3.2. Molecular evolutionary relationships*

All six isolates were bidirectionally sequenced, and the obtained sequences were registered in the NCBI GenBank with accession no. OK336713-18 *(Table 1).*

***Table 1. NCBI GenBank accession numbers of novel tobacco phytoplasma isolates obtained in this study, and their identity rates to reference isolates***

| Isolate | Province | Accession no | NCBI | *iPhyClassifier*-similarity coefficient |
|---|---|---|---|---|
| Tut-78 | Çanakkale | OK336713 | %99,84-MN047263 *Euscelis incisus* *Ca*. P. solani | 1.00 AF248959 *Catharanthus roseus* 16SrXII-A "Stolbur" (STOL11) |
| Tut-7 | Balıkesir | OK336714 | %99,78-MF503627 *Daucus carota* *Ca*. P. solani | |
| Tut-3 | Balıkesir | OK336715 | %99,87-MN047263 *Euscelis incisus* *Ca*. P. solani | |
| Tut-2 | Balıkesir | OK336716 | %99,84-MN047263 *Euscelis incisus* *Ca*. P. solani | |
| Tut-20 | Balıkesir | OK336717 | %99,92-MN047263 *Euscelis incisus* *Ca*. P. solani | |
| Tut-1 | Balıkesir | OK336718 | %99,79-MF503627 *Daucus carota* *Ca*. P. solani | |

Based on BLAST analysis, our nucleotide sequences shared nucleotide identity ranging from 99.79% to 99.92% compared with reference isolates of 16SrXII (blast.ncbi.nlm.nih.gov). *i*PhyClassifier analysis showed more specifically that all Turkish tobacco isolates had a 1.00 similarity coefficient to '*Candidatus* Phytoplasma solani' (16SrXII-A 'Stolbur') clone STOL11 (*Table 1*). Therefore, the nt sequences of 14 subgroup 16SrXII-A and 7 subgroup 16SrXII-B isolates, and also 20 group 16SrI, and 15 group 16SrVI isolates were retrieved from the NCBI GenBank to be compared to the six tobacco phytoplasma isolates. The six tobacco phytoplasma isolates were all positioned in a cluster together with other 14 subgroup 16SrXII-A isolates in the constructed phylogenetic tree, confirming that they are belonging to the subgroup (*Figure 2*).

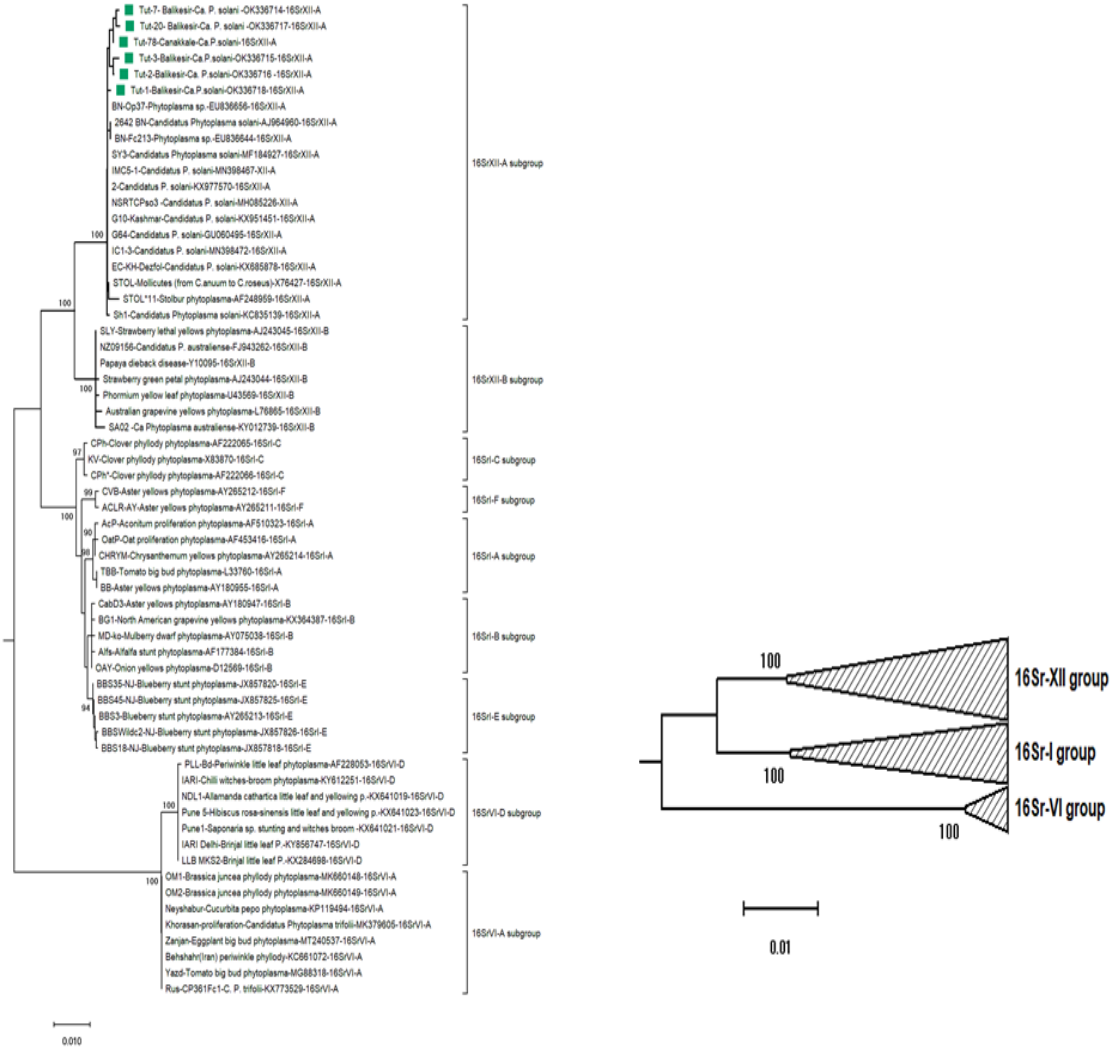


***Figure 2. Maximum-likelihood phylogenetic tree of nucleotide sequences of 16S rRNA gene fragment (1248 bp) of six novel tobacco isolates (OK336713-18) and 56 other phytoplasma isolates belonging to different groups were constructed using Kimura 2-parameter model and Gamma distributed rates among sites. Branches were supported by 1000 bootstrap replicates; only values greater than 90% were shown. The six novel tobacco isolates, marked with square dots, were clustered in subgroup 16Sr-XII-A***

Infection of tobacco crops by different groups of phytoplasma has been observed across the world (Mitrović and Duduk, 2011) but no comprehensive survey and molecular study on this subject has been reported. Moreover, only five isolates were available in the NCBI GenBank database on November 19, 2021, under the search terms "phytoplasma tobacco" and "phytoplasma nicotiana tabacum": Cuban tobacco phytoplasma (16SrII) isolate Granma (collected in Cuba, acc. no. EU328255), Tobacco phytoplasma (16SrI) strain Dhto1 (collected in India, acc. no. JF958126), and '*Ca*. P. solani' (16SrXII) strains 142/09, 159/10, and 284/09 (collected in Serbia, acc. no.

JQ730739, KC417488, and JQ730740). Therefore, the molecular characterization and population analysis presented in this current study could provide significant information on the tobacco infecting phytoplasma.

Molecular evaluation analysis showed that six new tobacco isolates were located in 16SrXII-A subgroups, with five of them being in a distinct subcluster, indicating that tobacco isolates might have unique genomic characteristics when compared to other 16SrXII-A isolates. The new tobacco isolates were positioned in a subcluster that was separated from two '*Ca*. P. solani' isolates (MN398467 and MN398472) from grape (*Vitis vinifera*) originated from the nearby Izmir province, indicating that genetic variations among Turkish isolates were probably more related to hosts than geographic locations. Although grape is also highly cultivated in Turkey (Bahar et al., 2019), the transmission of '*Ca*. P. solani' from other solanaceae to tobacco or vice versa is more likely, as tomato (*Solanum lycopersicum*) in Turkey has been known to also harbor '*Ca*. P. solani' isolates (Güller and Usta, 2020). The phylogenetic cladograms obtained from comparisons of three important ribosomal groups (16SrI/XII/VI) and their subgroups were similar to the topology of the three major phytoplasma subclades revealed in previous studies (Chung et al., 2013; Zhao et al., 2014).

A previous study showed that inoculation of apple proliferation phytoplasma (AP) (group 16SrX) into tobacco plants resulted in symptoms such as yellowing of leaves, reduced leaf size, and shoot proliferation (Lepka et al., 1999). Similarly, leaf yellowing, leaf blister, and growth disorder symptoms were also observed in naturally stolbur-infected tobacco in the present study (*Figure 1*).

### *3.3.* In silico-*RFLP analysis*

The virtual RFLP patterns of *Alu*I and *Mse*I restriction enzymes obtained using the 16S rRNA gene amplified with R16F2n/R16R2 universal primers from Turkish tobacco isolates showed high similarity with a 16SXII-A reference isolate (*Figure 3*). Additionally, digestion with commonly used 17 enzymes for each of the Turkish tobacco phytoplasma isolates showed that the profiles of all new isolates were similar to each other (*Figure 4*). Therefore, collective RFLP patterns derived by *iPhyclassifier* from the 16S rRNA gene fragment were consistent with results of phylogenetic analysis and revealed that the six-novel tobacco phytoplasma were members of subgroup 16SrXII-A.

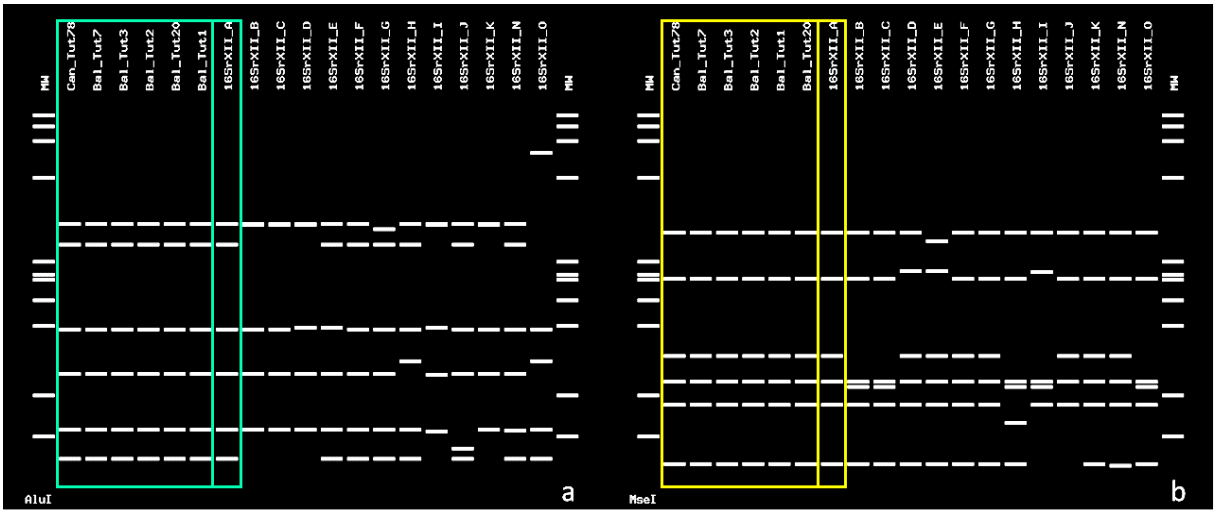


***Figure 3. In silico RFLP profile of the partial 16S rRNA gene (1.2 kb) constructed using the* iPhyclassifier *program (Zhao et al., 2013) from* AluI **(a)** *and* MseI *(b) endonucleases that distinguished (STOL) 16SrXII subgroups. Green and yellow boxes show similarity of 16Sr XII-A subgroup enzyme digest profile with Turkish tobacco isolates***

The results of the phylogenetic analysis were further supported by a computer-simulated virtual RFLP analysis, which can increase the accuracy of phytoplasma classification based on the 16S rRNA gene region (Zhao et al., 2009). As expected, the new tobacco isolates showed the exact *Alu*I and *Mse*I enzymes digest profile with a reference isolate of subgroup 16Sr XII-A. Additionally, a '*Ca*. P. solani' isolated from *Catharanthus roseus* (Periwinkle) in the USA (acc. no. AF248959) (Davis and Dally, 2001) showed an identical *in silico* RFLP profile

(similarity coefficient = 1.00) with all new tobacco isolates (*Table 1*); thus, they are probably the same phytoplasma isolate, but further study on other genome region is necessary to confirm this.

Stolbur phytoplasma is associated with a wide range of hosts such as celery, grapevine, strawberry, tomato, and lavender, and its presence in five major world continents has also been reported (Bertaccini et al. 2014). Likewise, it has been documented that this subgroup infects plants belonging to various families, such as carrot (Sertkaya, 2014), pistachio (Guldur et al., 2018), tomato (Güller and Usta, 2020), and grapevine (Şimşek and Güldür, 2021) grown in different regions of Turkey. However, to the best of our knowledge, this is the first record of infections caused by 16SrXII-A ribosomal subgroup phytoplasma in tobacco cultivation areas in Turkey.

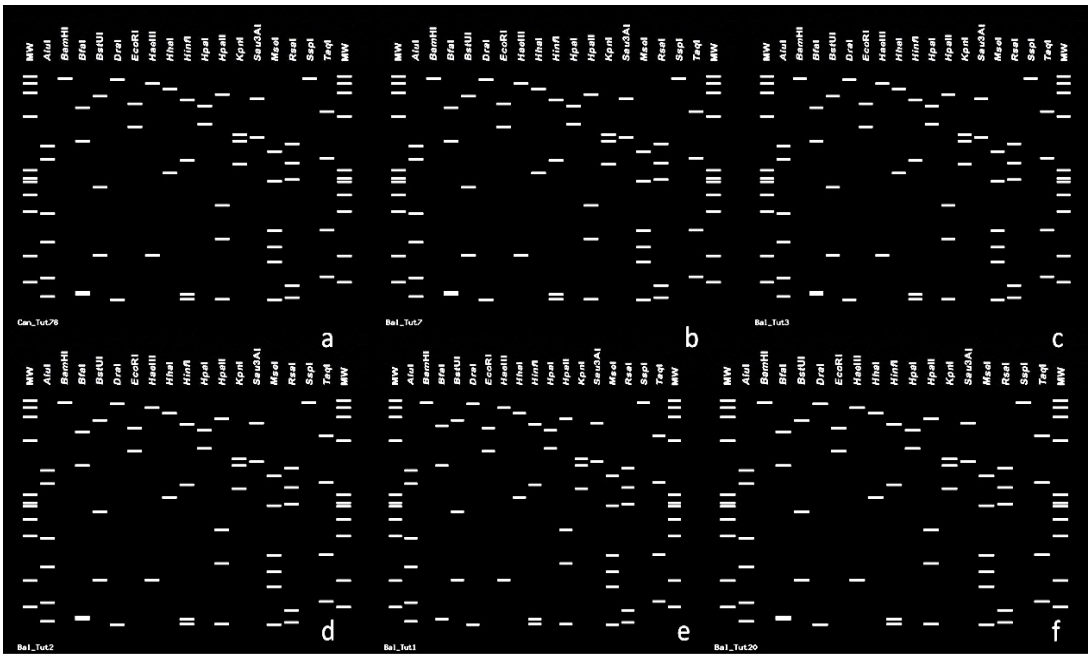


***Figure 4. In silico RFLP profile of the partial 16S rRNA gene (1.2 kb) of Novel isolates: a. Tut-78-Çanakkale, b. Tut-7- Balıkesir, c. Tut-3-Balıkesir, d. Tut-2-Balıkesir, e. Tut-1-Balıkesir, f. Tut-20-Balıkesir that were constructed using the iPhyclassifier program (Zhao et al., 2013) from 17 restriction enzymes***

### *3.4. Genetic distances*

The genetic distance values of analysed isolates within their respective groups 16SrI (AY), 16SrXII (STOL), and 16SrVI (CP) were all found to be very low. The genetic distance value between groups 16SrXII and 16SrVI (0.0709±0.0084) was estimated to be the highest in comparisons among three groups (*Table 2*), indicating that isolates of 16SrI were genetically more closely related to 16SrXII than 16SrVI.

***Table 2. Estimates of the average evolutionary divergence over sequence pairs of the 16S rRNA gene fragment (1248 bp) of phytoplasma isolates from different groups***

| Ribosomal Groups | 16SrI | 16SrXII | 16SrVI |
|---|---|---|---|
| 16SrI group | 0.01±0.00 | | |
| 16SrXII group | 0.0336±0.0045 | 0.01±0.00 | |
| 16SrVI group | 0.0642±0.0079 | 0.0709±0.0084 | 0.00±0.00 |
| Overall Mean Distance | 0.04±0.00 | | |

The relationships among the three groups were also examined in the genetic distance and genetic diversity analyses. 16SrXII had the greatest within-group variation compared to 16Sr-I and 16SrVI, according to both genetic distance and genetic diversity analyses. However, the genetic variation was minimal, as shown by the very low mean evolutionary distance as well as low π, S, and η values of each group (*Table 2* and *Table 3*). The evolutionary distance measures also determined that 16SrI had lower nt divergence to 16SrXII than 16SrVI (*Table*

*2*), which was in line with close positioning of 16SrI and 16SrXII in the main lineage of the phylogenetic tree, and clustering of 16SrVI in a separate lineage (*Figure 2*).

### *3.5. Genetic diversity and Neutrality analyses*

The π, S, and η values of each phytoplasma group were very low, but the metrics scored significantly for the combination of the three groups, indicating much lower genetic divergence at group level than at the genus level. 16SrXII group obtained higher π, S, and η values than two other groups, suggesting high diversity among its isolates, which includes the new six tobacco isolates (*Table 3*). The groups 16SrI and 16SrXII showed low nucleotide diversity (π = 0.00547 and 0.00860, respectively) but high haplotype diversity (Hd = 0.963 and 0.860, respectively). These results indicated that the populations have expanded recently and that there are haplotypes that are closely related to each other. Meanwhile, both π (0.00268) and Hd (0.590) values were very low for the group 16SrVI populations, showing high identity among isolates of the group (*Table 3*). The three neutrality tests assigned statistically insignificant negative values to 16SrI and 16SrXII groups while giving statistically significant and insignificant positive values to the 16SrVI group and the overall isolates examined in this study (*Table 4*).

***Table 3 Summary of genetic diversity analysis on the 16S rRNA gene fragment (1248 bp) of different phytoplasma groups***

| Ribosomal Groups | N | Pi | h | Hd | S | Eta |
|---|---|---|---|---|---|---|
| 16SrI Aster yellows | 20 | 0.00547 | 16 | 0.963 | 28 | 31 |
| 16SrXII Stolbur | 27 | 0.00860 | 15 | 0.860 | 41 | 41 |
| 16SrVI Clover proliferation | 15 | 0.00268 | 3 | 0.590 | 7 | 7 |
| All isolates | 62 | 0.04924 | 34 | 0.948 | 169 | 181 |

N: Number of Sequences, Pi: Nt diversity, H: No of Haplotypes, Hd: Haplotype diversity,S: Segregating sites, Eta: Total number of mutations

***Table 4 Summary of demography test statistics on the 16S rRNA gene fragment (1248 bp) of different phytoplasma groups***

| Ribosomal Groups | Fu and Li's *D** | Fu and Li's *F** | Tajima's *D* |
|---|---|---|---|
| 16SrI Aster yellows | -0.53302 ns | -0.63628 ns | -0.57907 ns |
| 16SrXII Stolbur | -0.63264 ns | -0.56594 ns | -0.15868 ns |
| 16SrVI Clover proliferation | 0.75006 ns | 1.23621 ns | 1.97479 ns |
| All isolates (16Sr-I/VI/XII) | 0.97365 ns | 1.71814 * | 2,13867 * |

Statistical significance: P > 0.10; 0.10 > P > 0.05; *, P < 0.05

Neutral selection results suggested demographic disparities among the three observed phytoplasma groups. The negative values obtained by 16SrI and 16SrXII groups indicate that the two populations are experiencing expanding or bottleneck selections, which could be driven by low-frequency polymorphism (new mutations) in the 16S rRNA gene fragment of their isolates. On the other hand, the 16SrVI population, which also was genetically divergent to 16SrI and 16SrXII, obtained positive values from the three neutrality tests. The results suggested that 16SrVI have evolved for a long time, thus undergoing balancing selections.

## 4. Conclusions

In our study, the phytoplasmas that cause infections in tobacco plants in Turkey are from the '*Ca*. P. solani' subgroup 16SrXII-A. By using 16S rRNA nt sequences of six positive samples and other global isolates, the population genetic structure of phytoplasmas of the 'stolbur' group was revealed. All the findings showed that the 16S rRNA gene region, which is used extensively in the detection and classification of phytoplasmas, is highly conserved; with the lowest nt identity being in the 16SrXII group. However, to better understand the epidemiology of the agent, host adaptation and/or selection, and to reveal the evolutionary mechanisms that govern these processes, more detailed studies and bioinformatic analyses with non-ribosomal phytoplasma genes (*Tuf, Vmp1, SecY,* etc.) are required in future studies.

## References

Akinyemi, I.A., Wang, F., Zhou, B., Qi, S., Wu, Q. (2016). Ecogenomic survey of plant viruses infecting tobacco by next generation sequencing. *Virology Journal* 13:1-12.

Bahar, E., Korkutal, İ., Şahin, N., Sağır, F.S., Kök, D., Ergönül, O., Uysal, T., Orhan Özalp, Z. (2019). Ganos Dağları doğal florasında bulunan kültür asmalarının (*Vitis vinifera* L.) moleküler ve ampelografik karakterizasyonu (in Turkish). *Journal of Tekirdag Agricultural Faculty* 16(1): 92-102.

Baric, S., Dalla-Via, I. (2004). A new approach to apple proliferation detection: A highly sensitive Real-Time PCR assay. *Journal of Microbiology Methods* 57:135-145.

Bertaccini, A., Duduk, B., Paltrinieri, S., Contaldo, N. (2014). Phytoplasmas and phytoplasma diseases: a severe threat to agriculture. *American Journal of Plant Sciences* 5:1763-1788.

Bertaccini, A., Lee, I.M. (2018). Phytoplasmas: An Update: Characterisation and Epidemiology of Phytoplasma - Associated Diseases. In: Rao GP, Bertaccini A, Fiore N, Liefting L, editors. Phytoplasmas: Plant Pathogenic Bacteria-I. Singapore: Springer;. p. 1-29

Chung, W.C., Chen, L.L,, Lo, W.S., Lin, C.P., Kuo, C.H. (2013). Comparative analysis of the peanut witches'-broom phytoplasma genome reveals horizontal transfer of potential mobile units and effectors. *PLoS One* 8(4):e62770. doi:10.1371/journal.pone.0062770.

Çağlar, B.K., Elbeaino, T., Küsek, M., Pehlivan, D., Fidan, H., Portakaldali, M. (2010). Stolbur Phytoplasma infections in potato and tomato plants from different locations in Turkey. *Journal of Turkish Phytopathology* 39(1-3):1-8.

Çulal-Kiliç, H., Çıkrıkçı, M.Ö., Yardımcı, N. (2017). Determination of Tobacco mosaic virus in tobacco fields in Denizli province, Turkey. *Scientific Papers. Series A. Agronomy* 60:215-219.

Davis, R.E., Dally, E.L. (2001). Revised subgroup classification of group 16SrV phytoplasmas and placement of flavescence dorée-associated phytoplasmas in two distinct subgroups. *Plant Disease* 85:790-797.

Erdem, N. (2010). *Detection of viruses infecting tobacco (Nicotiana tabacum L.) in Samsun province (in Turkish)*. Master Thesis. Samsun: Ondokuz Mayis University. 63 pp.

Fu, Y.X., Li, W.H. (1993). Statistical tests of neutrality of mutations. *Genetics* 133(3):693-709.

Guldur, M.E., Simsek, E., Caglar, B.K., Dikilitas, M., Gumus, H., Ayvaci, H. (2018). First report of a 'Candidatus Phytoplasma solani'-related strain (16SrXII-A subgroup) associated with pistachio leaf scorch disease in Turkey. *New Disease Reports* 38:4.

Gundersen, D.E., Lee, I.M. (1996). Ultrasensitive detection of phytoplasmas by Nested-PCR assays using two universal primer pairs. *Phytopathologia Mediterranea* 35(3):144-151.

Güller, A., Usta, M. (2020). Stolbur and clover proliferation phytoplasma infections in tomato from Bingöl province, Turkey. *Türk Tarım ve Doğa Bilimleri Dergisi* 7(4): 855–866.

Karanfil, A., Sarı, M., Korkmaz, S. (2020). First report of tobacco mild green mosaic virus in Turkey. *Journal of Plant Pathology* 102:547. doi:10.1007/s42161-019-00436-x.

Kimura, M. A (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* 16:111-120.

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Molecular Biology and Evolution* 35(6):1547-1549.

Lepka, P., Stitt, M., Moll, E., Seemüller, E. (1999). Effect of phytoplasmal infection on concentration and translocation of carbohydrates and amino acids in periwinkle and tobacco. *Physiological and Molecular Plant Pathology* 55(1):59-68.

Lherminier, J., Benhamou, N., Larrue, J., Milat, M.L., Boudon-Padieu, E., Nicole, M., et al. (2003). Cytological characterization of elicitin-induced protection in tobacco plants infected by Phytophthora parasitica or phytoplasma. *Phytopathology* 93(10):1308-1319.

Li, R., Mock, R., Huang, Q., Abad, J., Hartung, J., Kinard G. (2008). A reliable and inexpensive method of nucleic acid extraction for the PCR-based detection of diverse plant pathogens. *Journal of Virological Methods* 154(1-2):48-55.

Marcone, C. (2014). Molecular biology and pathogenicity of phytoplasmas. *Annual Applied Biology* 165(2):199-221.

Mitrović, J., Duduk, B. (2011). Occurrence of a new stolbur strain in tobacco in Serbia. *Bulletin of Insectology* 64(Supplement):107-108.

Rozas, J., Ferrer-Mata, A., Sánchez-DelBarrio, J.C., Guirao-Rico, S., Librado, P., Ramos-Onsins, S.E., Sánchez-Gracia, A. (2017). DnaSP 6: DNA sequence polymorphism analysis of large data sets. *Molecular Biology and Evolution* 34:3299-3302.

Sertkaya, G., (2014). Hatay ili havuç alanlarında fitoplazmaların araştırılması (in Turkish). The 5th Turkish Plant Protection Congress. Antalya, Turkey; 3-5 February 2014.

Stanković, I., Bulajić, A., Vučurović, A., Ristić, D., Milojević, K., Berenji, J., et al. (2011). Status of tobacco viruses in Serbia and molecular characterization of Tomato spotted wilt virus isolates. *Acta Virology* 55:337-347.

Şimşek, E. Güldür, M.E. (2021).Detection and molecular characterization of phytoplasmas based on 16s rDNA gene region by phylogenetic and in silico RFLP analysis of local grapevine cultivars in Şanlıurfa and Adıyaman. *Harran Tarım ve Gıda Bilimleri Dergi*si 25(2):204-213.

Tajima, F. (1989). Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* 123(3):585-595.

Temel, U., Öztekin, Y.B. (2020). Tokat ilinde kullanılan bitki koruma makinelerinin ürün güvenliği açısından değerlendirilmesi (in Turkish). *Journal of Tekirdag Agricultural Faculty* 17(3): 276-284. doi: 10.33462/jotaf.639208

TSI (Turkish Statistical Institute) (2021). Crop Production Statistics. https://biruni.tuik.gov.tr/medas/?kn=92&locale=en. (accessed date: 19.11.2021).

Usta, M., Güller, A., Günay, A. (2020). The molecular characterization of the coat protein sequence and differentiation of CMV-subgroup I on tobacco from native flora in Turkey. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca* 48(2), 523-534.

Usta, M., Güller, A., Sipahioğlu, H.M. (2018). Molecular analysis of 'Candidatus Phytoplasma trifolii' and 'Candidatus Phytoplasma solani' associated with phytoplasma diseases of tomato (PDT) in Turkey. *International Journal of Agriculture and Biology* 20(9):1991-1996.

Weintraub, P.G., Wilson, M.R. (2009). Control of phytoplasma diseases and vectors. In: Weintraub PG, Wilson MR, editors. Phytoplasmas: genomes, plant hosts and vectors. Wallingford, United Kingdom: CAB International; p. 233-249.

Wolf, F.A. (1949). Turkish or Oriental Tobacco. Economic Botany.;3(1):32-41. https://www.jstor.org/stable/4251921.

Yilmaz, S., Caglar, B.K., Djelouah, K. (2019). Molecular characterization of phytoplasma diseases of pepper in Turkey. *Journal of Phytopathology* 167(9):479-483.

Yürekli, A., Önder, Z., Elibol, M., Erk, N., Cabuk, A., Fisunoglu, M., et al. (2010). The Economics of Tobacco and Tobacco Taxation In Turkey. International Union Against Tuberculosis and Lung Disease. https://www.tobaccofreekids.org/assets/global/pdfs/en/Turkey_tobacco_taxes_report_en.pdf. (accessed date: 22.11.2021).

Zhao, Y., Davis, R.E., Wei, W., Shao, J., Jomantiene, R. (2014). Phytoplasma genomes: evolution through mutually complementary mechanisms, gene loss and horizontal acquisition. In: Gross D, Berlin C, editors. Genomics of Plant-Associated Bacteria. Heidelberg: Springer-Verlag; p. 235-271.

Zhao, Y., Wei, W., Lee, I.M., Shao, J., Suo, X., Davis, R.E. (2009). Construction of an interactive online phytoplasma classification tool, iPhyClassifier, and its application in analysis of the peach X-disease phytoplasma group (16SrIII). *International Journal of Systematic and Evolutionary Microbiology* 59(10):2582-2593.

Zhao, Y., Wei, W., Lee, I.M., Shao, J., Suo, X., Davis, R.E. (2013). The iPhyClassifier, an Interactive Online Tool for Phytoplasma Classification and Taxonomic Assignment. In: Dickinson M, Hodgetts J, editors. Methods in Molecular Biology: Phytoplasma. Totowa, NJ: Humana Press; p. 329-338.