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Research Article/Araştırma Makalesi

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

Şanlıurfa ve Adıyaman illerinde yerel asma çeşitlerindeki fitoplazmaların 16s rDNA gen bölgesine dayalı filogenetik ve in silico RFLP analizleri ile tespiti ve moleküler karakterizasyonu

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ABSTRACT

During the autumn seasons of 2016-2019, several surveys were carried out in grapevine (*Vitis vinifera* L.) cultivation areas in Şanlıurfa and Adıyaman provinces of South-eastern part of Turkey, to identify grapevine plants exhibiting phytoplasma-like symptoms including yellowing, small leaf formation, chlorosis, short internodes, severe redness and inward curling and to detect and characterize the causal agent responsible for these symptoms. Purified DNA obtained from collected samples was examined for the highly conserved phytoplasma 16S rDNA gene via nested-PCR with the universal phytoplasma-specific primer sets. DNA amplification via nested-PCR/RFLP analyses with some restriction enzymes confirmed the suspected correlations between the disease symptoms observed and phytoplasma presence in the samples. F2n/R2 amplicons of the phytoplasma strains obtained from the samples showed 99.99% homology with each other and 99% homology with phytoplasma DNA partial sequences belonging to some groups deposited in the GeneBank database. Sequencing and phylogenetic analysis revealed that collected isolates have 99% sequence identity with '*Candidatus* phytoplasma solani' (16SrXII-A) and '*Ca*.P. asteris' (16SrI-B). According to our knowledge, the results of this study is the first report of the phylogenetic relationship of phytoplasmas infecting different grapevine cultivars based on 16S rDNA gene. Genetic diversity of genes other than 16S rDNA of the pathogen causing these infections is under study.

Key Words: Grapevine yellows, Phytoplasma disease, Şanlıurfa, Nested-PCR, Grapevine

ÖZ

Türkiye'nin Güneydoğusundaki Şanlıurfa ve Adıyaman illerinde bağ (*Vitis vinifera* L.) üretim alanlarında 2016-2019 sonbahar sezonlarında, yapraklarda sararma, küçük yaprak oluşumu, kloroz, boğumlar arasında kısalma, şiddetli kızarmalar ve içe doğru kıvrılmalar gibi fitoplazma benzeri semptomlar sergileyen asma bitkilerindeki belirtilerin etmenini belirlemek ve karakterize etmek için çeşitli sürveyler yapılmıştır. Toplanan örneklerden elde edilen saflaştırılmış DNA, fitoplazmalara özgü üniversal primer setleri ile nested-PCR yöntemi ile yüksek oranda korunmuş fitoplazma 16S rDNA geni üzerinden incelenmiştir. Nested-PCR ile DNA amplifikasyonu ve RFLP analizleri ile örneklerdeki gözlenen simptomlar ve fitoplazma varlığı arasındaki beklenen korelasyon doğrulanmıştır. Örneklerden elde edilen fitoplazma türlerinin F2n / R2 amplikonları, birbirleriyle %99.99 homoloji göstermiş ve NCBI GeneBank veri tabanında bulunan bazı gruplara ait fitoplazmalar ile de %99 sekans homolojisi göstermiştir. DNA dizileme ve filogenetik analizler, toplanan izolatlardaki etmenlerin "C*andidatus* fitoplazma solani" (16SrXII-A) ve "*Ca*.P. asteris'in (16SrI-B) olduğunu ortaya koymuştur. Bildiğimiz kadarıyla bu, 16S rDNA genine dayalı farklı yerel asma çeşitlerini enfekte eden fitoplazmaların filogenetik ilişkisinin ilk raporudur. Bu tip enfeksiyonlara neden olan patojenin 16S rDNA dışındaki genlerin genetik çeşitliliği araştırma aşamasındadır.

Anahtar Kelimeler: Asma sarılığı, Fitoplazma hastalığı, Şanlıurfa, Nested-PCR, Asma

Introduction

Phytoplasmas are mollicutes that lack of cell wall and only limited to live in plant phloem infecting a wide range of insect and plant species worldwide (Bertaccini et al., 2014). Their identifications were previously based on electron microscopy and/or symptomatically observations, however, during the last three decades, 16S rDNA sequences of phytoplasmas amplified by PCR has played a pivotal role in the accurate detection and classfication of different isolates and the discovery of novel phytoplasmas in microbiology laboratories (Lee et al., 1998; Bertaccini and Lee, 2018). Among the plant species suitable host for phytoplasmas, the grapevine is considered to be one of the major fruit crops worldwide based on cultivated area and economic value worldwide (Pierro et al., 2019). Diseases caused by phytoplasmas generally described as "Grapevine yellows (GY)" have been reported in many viticultural areas worldwide, including the Americas, Africa, Australia, Asia and Europe (Constable and Bertaccini, 2017).

Grapevine is a plant species that is one of the oldest cultivated one for Anatolian farmers, therefore, Turkey is known as the origin of viticulture and wine-making (Gorny, 2003; Gokbayrak and Soylemezoglu, 2010). Although several '*Candidatus* phytoplasma' species belonging to different taxonomic groups infecting grapevine plants have been identified worldwide, only a few 16Sr-group including 16SrI, 16rV, 16SrIX and 16SrXII were identified and reported in Turkey (Canik et al., 2011; Ertunc et al., 2015). 16SrI, 16SrVII-A and 16SrII-B group phytoplasmas were also reported in grapevine exhibiting leaf scorch, leaf yellowing, redding and decline symptoms in Iran, recently (Zamharir et al., 2017; Babaei et al., 2019). Irregular leaf yellowing symptoms for white varieties and reddening symptoms for red varieties, chlorosis, necrosis and leaves curling backward were also observed in local vineyards of Şanlıurfa and Adıyaman provinces in Turkey. These disease symptoms are consistent with the symptoms associated with phytoplasmas according to Dermastia et al. (2017) and many other previously reported studies. Therefore, many surveys have been conducted in the viticultural areas in these two provinces to verify phytoplasma presence and identity. Etiological studies, identifying and initial characterization of causal agents constitute the most significant step for the control and/or management of the disease. Therefore, this study was carried out to detect and identify possible causal agent(s) for the symptoms observed on local grapevine varieties.

Materials and Methods

Surveys and plant material

Several vineyards in Şanlıurfa and Adıyaman provinces with/without a history of crop losses presumably due to virus or phytoplasma disease symptoms were surveyed for the detection of possible phytoplasma occurrences. During the surveys, grapevine leaves and shoots were collected from different grapevine cultivars (*Vitis vinifera* L.) (Fig. 1) evidencing putative symptoms associated with the '*Candidatus* Phytoplasma spp.' (Dermastia et al., 2017). Mostly, symptomatic plant samples were collected, however, in some cases, symptomless plant tissues were also collected if phytoplasmas are existed in asymptomatic grapevine plants.

DNA isolations and PCR

Total nucleic acids from samples were isolated from 1 g of fresh grapevine leaf midrib of symptomatic and asymptomatic plants as described by Ahrens and Seemüller (1992) with the minor modifications. This method is based on the enrichment of DNA amount during the isolation process. Leaf midribs were ground in lysis buffer (20 mmol L^{-1} EDTA, 1.4 mol L^{-1} NaCl, 100mmol L^{-1} Tris–HCl pH 8.0, 2% w/v cetyltrimethylammonium bromide (CTAB), and 0.2% w/v 2-mercaptoethanol) and the extracts were incubated at 65°C for 30 min. An equal volume of chloroform-isoamyl alcohol (24:1) was added to the lysis buffer (CTAB) and strongly

mixed centrifuged at 10.000 *g* for 10 min. Chloroform-isoamyl alcohol adding step was repeated twice. Supernatant was precipitated overnight at -20°C with 0.6 volume of isopropanol. 70% ethanol was used to wash the pellet obtained following centrifugation at 8.000 *g* for 10 min. Then the pellets were vacuum-dried and suspended in 50 µl Tris-EDTA (TE) buffer. DNA concentrations and purity were estimated by using a spectrophotometer according to standard techniques and procedures (Desjardins and Jonklin, 2010). Isolated DNAs were then used as template for direct PCR analysis with the primers showed in Table 1.

Table 1. Universal primer sets used for PCR analyses for detection of phytoplasmas

Primers	Universal / Group Specific*	Primer sequences (5'-3')	Expected band size
R16F1 R16R0	Universal	AAGACGAGGATAACAGTTGG GGATACCTTGTTACGACTTAACCCC	1.4 kb
R16F2n R16R2	Universal	GAAACGACTGCTAAGACTGG TGACGGGCGGTGTGTACAAACCCCG	1.2 kb
R16(I)F1 R16(I)R1	G.S.	TAAAAGACCTAGCAATAGG CAATCCGAACTAAGACTGT	1.1 kb
M1 M2	Universal	GTCTTTACTGACGCTGAGGC CTTCAGCTACCCTTTGTAAC	0.5 kb
R16(V)F1 R16(V)R1	G.S.	TTAAAAGACCTTCTTCGG TTCAATCCGTACTGAGACTACC	1.1 kb

*G.S.: Group specific

Direct-PCR analyses were performed with R16F1/R0 universal primer pair (Lee et al., 1995) amplifying a 1.4 kb product. Direct-PCR products were then diluted as 1/50 to 1/100 and used as DNA template for a nested-PCR ,second-round PCR, using R16F2n/R2 and R16(I)F1/R1 primers amplifying an internal fragment of 1.2 and 1.1 kb in size from the 16S rDNA gene, respectively (Gundersen and Lee, 1996). Specific DNA fragments 1.1 kb in size approximately were amplified in second nested-PCR with R16(I)F1/R1 primers that allow group-specific phytoplasma (16SrXII and 16SrI) recognition according to Lee et al. (1995). M1/M2 internal primer pair was used for testing the specificity and sensitivity (Gibb et al., 1995). PCR amplifications were performed in total 50 µl reaction mixtures each containing 1 µl of template DNA, 5 µl of 10X Dream Taq Green buffer, 1 µl of dNTPs (10 mM), 1 µl of forward primers (10 pmol) and 1 µl of reverse primers (10 pmol), 1.25U Dream Taq DNA polymerase (5U/µl) (Thermo Fisher Scientific) and 40.75 µl sterile water. Applied Biosystems Veriti 96™ Thermal Cycler was used for the PCR reactions. PCR products were then electrophoresed in agarose gel (1%), stained with ethidium bromide, and photographed under UV transillumination (312 nm).

Sequence and phylogenetic analysis

In the detection of the taxonomic position of grapevine phytoplasmas isolated from local varieties, nested-PCR products obtained from positive samples were purified and directly subjected for automated sequencing in both directions with the specific PCR primers used in each amplification (ABI 3130xl Genetic Analyzer, Applied Bio. Medsantek Ltd. Co, Turkey). The sequences were subjected to a BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi) NCBI search for most similar sequences in the database (12.03.2019). Sequences were trimmed and assembled using computer-assisted software, MEGA7: Molecular evolutionary Genetics Analysis Version 7.0 (Kumar et al., 2016). Obtained 16s rDNA sequences were then aligned with Cluster X analysis (Thompson et al., 1997).

Restriction fragment length polymorphism (RFLP) analysis and in silico enzyme digestions

RFLP analysis of F2n/R2 products was used for the initial classification of the phytoplasmas from nested-PCR positive samples (Duduk et al., 2013). In wet RFLP analysis, F2n/R2 products were directly subjected to enzyme digestions using *Eco*RI, *Hha*I, *Hpa*II, *Rsa*I and *Taq*I for preliminary

classification of phytoplasmas according to manufacturer's instructions (ThermoFisher Scientific, USA). In order to make a more comprehensive analysis of isolates and group/subgroup identification *in silico* restriction analysis were carried out using *i*PhyClassifier and pDRAW32 (AcaClone Software; http://www.acaclone.com). Virtual RFLP patterns were obtained from F2n/R2 product sequences of five representative sample by using *i*PhyClassifier (Zhao et al., 2009) and the sequences were subjected virtual enzyme digestions using 17 restriction enzymes [RsaI, SspI, and TaqI, BfaI, BstUI (ThaI), AluI, BamHI, DraI, HinfI, HpaI, EcoRI, HaeIII, HhaI, Sau3AI (MboI), HpaII, KpnI, MseI] that are used for differentiation of ribosomal groups and subgroups in virtual RFLP analysis (Lee et al., 1998).

Results and Discussion

Survey results and phytoplasma detections

During surveys, the sporadic occurrence of symptomatic plants was noted in Şanlıurfa and Adıyaman viticultural regions based on visual inspection. The most commonly observed symptoms (yellow leaf tissue becoming necrotic, small leaf formation, chlorosis, short internodes, severe redness and inward-curling) occured in mid- and late summer. Different symptom patterns were noticed in different cultivars; irregular leaf yellowing in white cultivars (especially in cultivar Çiloreş) and reddening of leaves in red cultivars such as Hönüsü (Figs 1A and B). However, according to visual observations, symptomatic differences caused by different phytoplasmas were not observed. These varieties are the most commonly used grapevines in these regions (Gursoz, 1993; Bekisli et al., 2015).

Nested-PCR analysis using for identification of phytoplasmas confirmed the expected interrelationship between symptoms observed in field and phytoplasma infections. Totally, 25 of the 137 grapevine leaves (with petiol) samples exhibiting grapevine yellows type disease symptoms were confirmed for phytoplasma infection via Nested-PCR.

The infection rate of grapevine phytoplasmas in Şanlıurfa and Adıyaman local varieties were found as 22.1 % and 9.52 %, respectively as shown in Table 2.

Table 2. Distribution of the phytoplasmas identified in surveyed viticultural areas

Region		Number of Samples Collected	Number of Positive Samples	Total Infection Rate (%)	Ribosomal Group		
					XII-A	I-B	Others
Şanlıurfa	Hönüsü	53	9	16.98	3	6	
	Çiloreş	30	10	33.3	9	1	
	Others	12	2	16.6	-	1	1
Adıyaman	Hönüsü	18	-	-	-	-	
	Çiloreş	20	4	20	3	1	
	Others	4	-	-			

No amplification signal was observed in healthy grapevine and negative control samples without DNA template in the first-round PCR products using F1/R0 primers. Nested-PCR amplification with M1/M2, R16(I)F1/R1 and R16F2n/R2 primers produced the expected amplicon length of about 0.5 kb, 1.1 kb and 1.2 kb (data not shown), respectively (Figure 2). No amplification was observed with the primers R16(V)F1/R1.

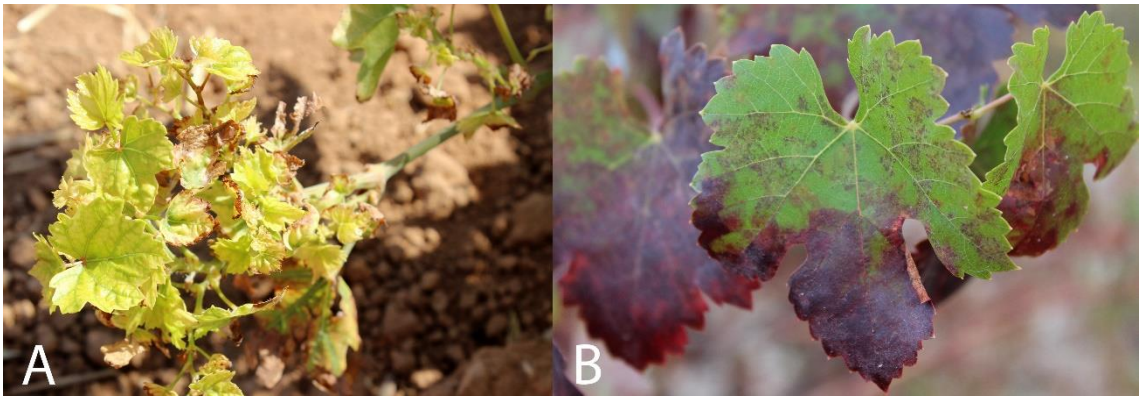

Figure 1. Disease symptoms caused by phytoplasmas in Şanlıurfa, A: leaf tissue chlorosis becoming necrotic in white cultivar Çiloreş, B: Severe reddening of leaves in red variety Hönüsü.

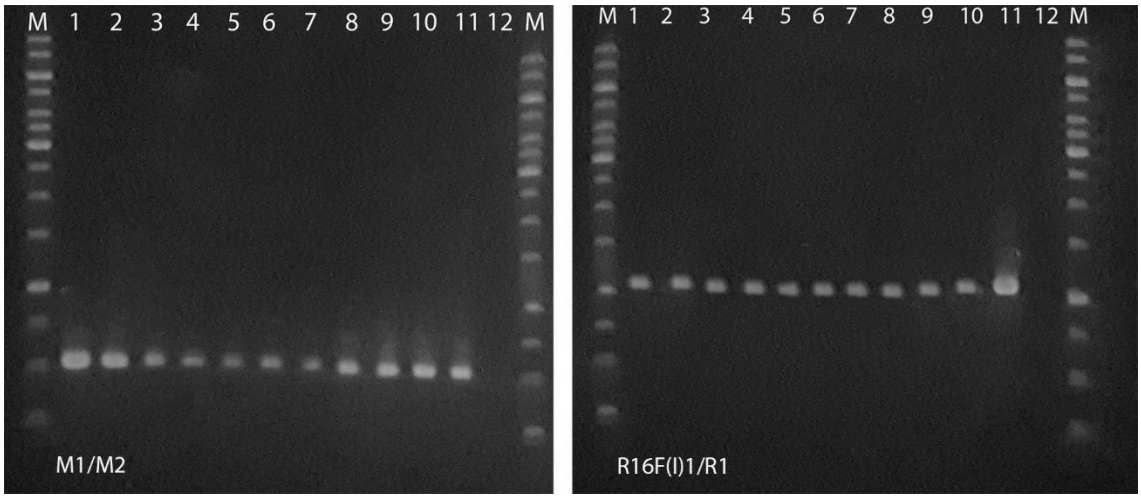

Figure 2. Agarose gel electrophoreses (%1) of phytoplasma 16S rDNA PCR products from Şanlıurfa and Adıyaman samples (1-10) and positive controls (11). Detected fragments were obtained with primer pairs: M1/M2 ($16R_{738f}$ /$16R_{1232r}$) and R16(I)F1/R1 and negative controls (12), M - marker GeneRuler 1 kb DNA Ladder (ThermoFisher Scientific).

RFLP and cladistic analyses

Amplified phytoplasma fragments obtained from local grapevine samples were subjected to the wet and *in silico* RFLP analyses with some distinctive RFLP enzymes In group-subgroup recognition of phytoplasmas.

The RFLP patterns of the 1.2 kbp 16SrDNA amplicons of symptomatic grapevines from the surveyed fields were mostly identical to each other and consisted with 16SrI and 16SrXII phytoplasma sub-group patterns (Lee et al., 1998).

Subgroup classification of phtoplasmas on iPhyClassifier

Samples coded as Grs19 and Grs72 were determined as representative samples according to wet RFLP results (data not shown). According to *i*PhyClassifier 16S rDNA sequences of GrS19 and GrS72 shares 99.7% and 99.5% similarities with that of the reference strains of '*Candidatus* Phytoplasma solani' with AF248959 accesion number and *'Candidatus* Phytoplasma asteris' with M30790 accesion number, respectively. So, these results suggest that the phytoplasmas under the study are *Candidatus* Phytoplasma solani' -related strain and '*Candidatus* Phytoplasma asteris'-related strains.

In silico RFLP patterns obtained from the 16S rDNA F2n/R2 fragments of GrS19 and GrS72 are found identical (with similarity coefficient 1.00) to 16Sr group XII, subgroup A (Accesion number of GenBank reference strain: AF248959) and the reference pattern of 16Sr group I, subgroup B (Accesion number of GenBank reference strain: AP006628), respectively. These results confirmed that the phytoplasmas under this study are members of 16SrXII-A and 16SrI-B, respectively.

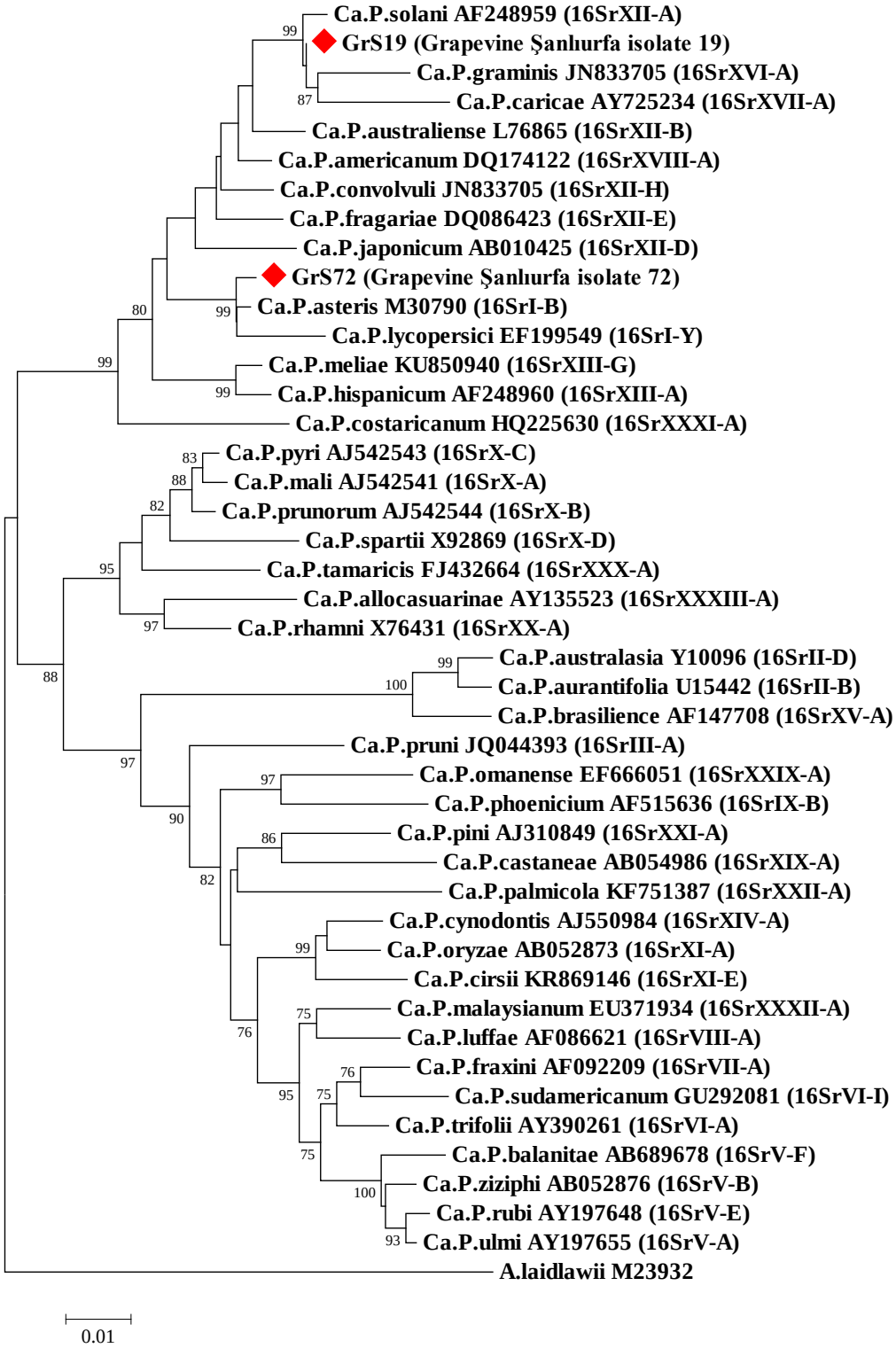

Figure 3. Phylogenetic tree using of the neighbour-joining method, based on 16S rDNA sequence of all phytoplasma species those of selected phytoplasma strains deposited in GenBank.

Computer-simulated *in silico* PCR-RFLP analyses of GrS19 and GrS72 based on 16SrDNA sequences with commonly used 17 RFLP enzymes [RsaI, SspI, and TaqI, BfaI, BstUI (ThaI), AluI, BamHI, DraI, HinfI, HpaI, EcoRI, HaeIII, HhaI, Sau3AI (MboI), HpaII, KpnI, MseI] showed identical patterns for restriction sites (similarity coefficient 1.00) to 16SrXII and 16SrI group reference phytoplasma strains with the accesion numbers of AF248959 and M30790, respectively (Figure 4).

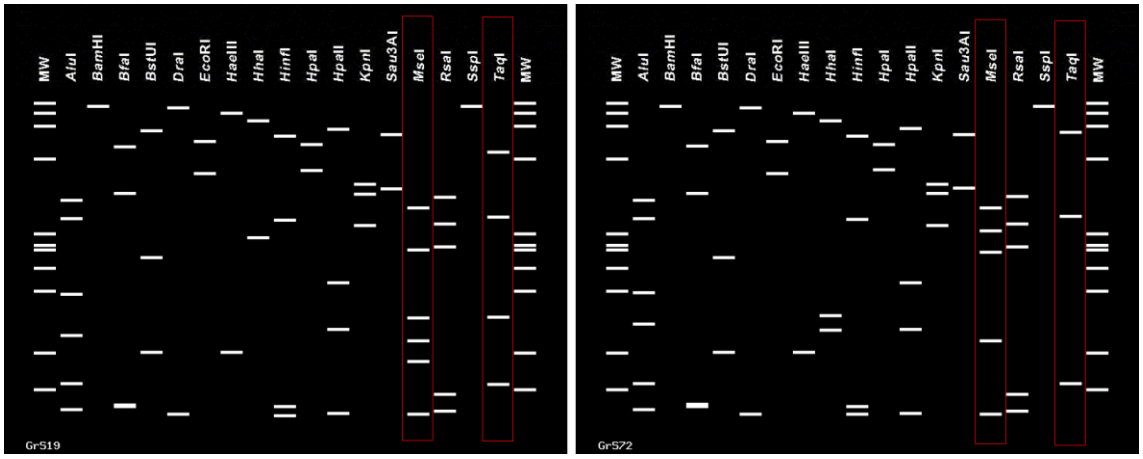

Figure 4. Virtual F2n/R2 RFLP profiles of 16Sr gene fragments obtained from 16SrXII-A subgroup phytoplasma strain (GrS19) and 16SrI-B subgroup phytoplasma strain (GrS72) from Şanlıurfa. Red boxes show that a clear separation into two distinct representative isolates and these patterns were also obtained from wet RFLP analysis (data not shown). MW: Marker, Φx174 DNA profile digested with enzyme *Hae*III.

Wet and Virtual RFLP analyses enabled that group/subgroup classification of isolates by comparison with reference strains for group and subgroup recognition. Also, virtual digestion patterns generated by pDraw32 software showed in Figure 5 supported these results. 16S rDNA partial gene sequences of phytoplasmas were aligned by CLUSTAL X software and approximately 1.2 kb DNA fragments were used for *in silico* digestions and virtual gel plotting. Phytoplasma strains obtained from diseased grapevine plants were classified into 2 groups according to *in silico* RFLP digestions and similarity coefficient calculations.

Based on conservative 16S rDNA gene analysis by nested-PCR confirmed phytoplasma presence in some local grapevine varieties exhibiting phytoplasma-like disease symptoms collected from Şanlıurfa and Adıyaman Provinces in Turkey. Result of this study confirmed that Grapevine Yellows diseases in one of the most viticultural areas in Şanlıurfa and Adıyaman provinces are mostly associated with *'Candidatus* Phytoplasma solani' (16SrXII-A) and *'Candidatus* Phytoplasma asteris' (16SrI-B). According to the results of this study, two '*Ca*. Phytoplasma' species associated with grapevine cultivated in Şanlıurfa and Adıyaman provinces were detected by nested PCR amplification of 16S rDNA gene region. The total infection rate of phytoplasmas among all collected samples with disease symptoms was found at 18.25%. The detection percentage of phytoplasmas in symptomatic plant samples in Şanlıurfa and Adıyaman provinces were found as 22.1% and 9.5%, respectively.

The disease of the grapevine caused by '*Candidatus* Phytoplasma solani' – related strains (16SrXII-A) is called as "Bois noir" (Quaglino et al., 2013) and Bois noir was reported in many countries including some European countries and Turkey (Constable and Bertaccini, 2017; Ertunc et al., 2015). On the other hand, Aster yellows phytoplasmas (16SrI group) are one of the largest group and are of genetic diversity when compared to other phytoplasma groups (Lee et al. 2004). Recently, these phytoplasma species had already been reported in Iran and Turkey (Ertunc et al., 2015; Babaei et al., 2019).

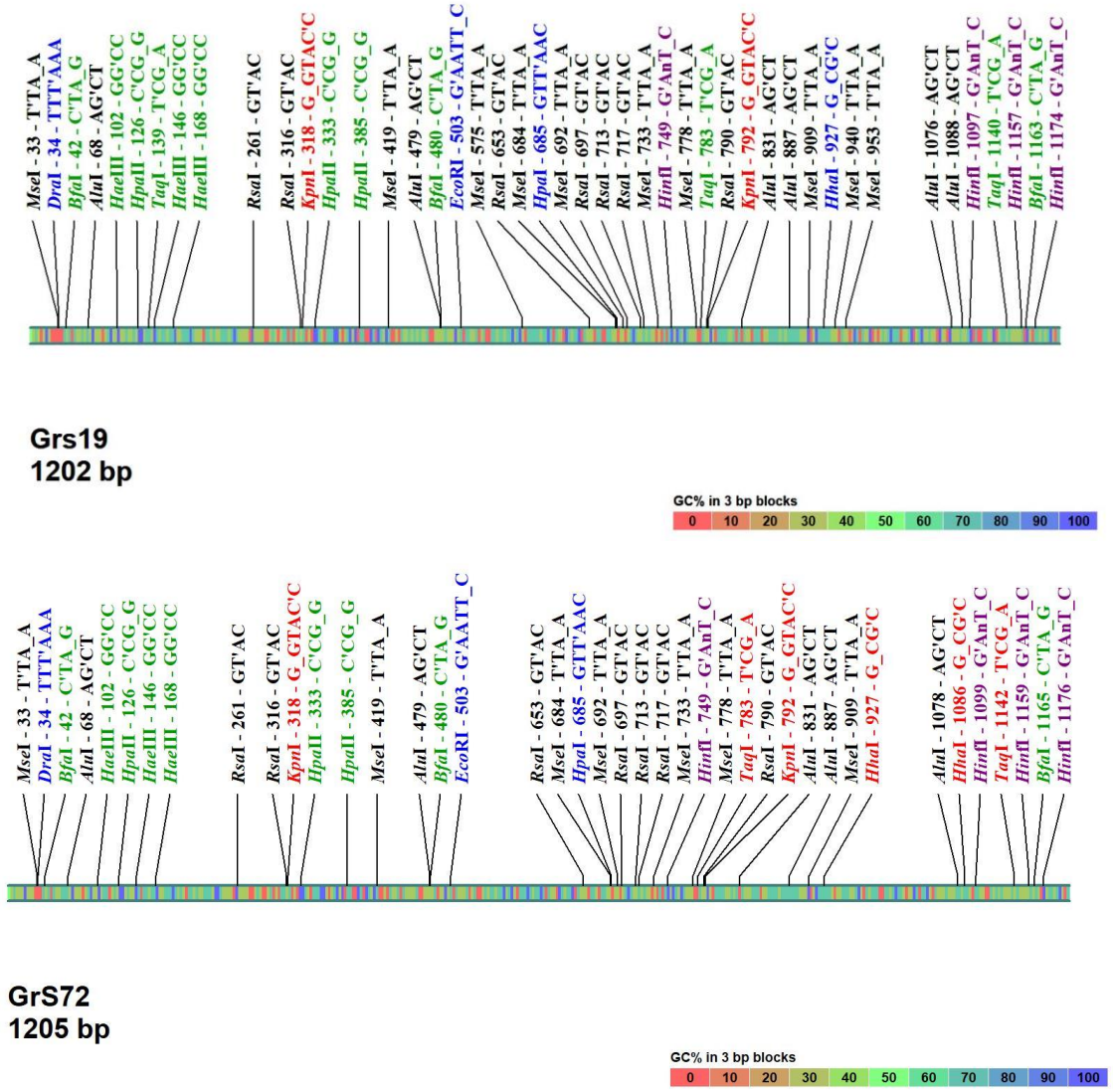

Figure 5. Patterns of restriction sites of GrS19 and GrS72 phytoplasma isolates based on partial 16S rDNA gen sequence. Maps were generated by using pDRAW32 DNA analysis software (by AcaClone software, http://www.acaclone.com/). Comparison of recognition sites for restriction endonucleases AluI, BfaI, DraI, EcoRI, HaeIII, HhaI, HinfI, HpaI, HpaII, KpnI, MseI (Tru1I), RsaI, and TaqI.

This study thus provided new information for the epidemiological distribution of 16SrXII-A and 16SrI-B subgroup phytoplasmas in Turkey. The motion of propagation cuttings may distribute infections to other viticultural areas in other geographic lands as grapevine is propagated by using of stem cuttings. Fortunately, no FD infected samples were found in the region, however, further studies are necessary to monitoring phytoplasma and virus diseases in viticultural areas because in the presence of disease-transmitting insect vector, detected diseases may limit grape production in the region.

Determination of possible alternative weed hosts, disease-transmitting insect species and phytoplasma phylogeny based on genes other than 16s rDNA genes are under investigation.

Conclusions

With this study, only 16SrI-B and 16SrXII-A subgroups were identified in 9 and 16 positive samples collected different small and mid-scale family vineyards. In these fields, production is mostly performed based on traditional methods, therefore, in some vineyards, weed control is rarely carried out. The level of alternative weed hosts should be determined in the region, because in the presence of disease-transmitting insects, the devastating effect of the disease may increase. Moreover, new data obtained from studies like our investigation will greatly contribute to knowledge of interactions between phytoplasmas and their weed hosts in viticultural areas. The symptom expressing but found as

phytoplasma-negative samples may be caused by viruses. For sustainable disease management, it is necessary to identify the causal agent of similar symptom expressing plants, phytoplasma epidemiology with potential insect vectors and to determine the regional distribution of the diseases caused by phytoplasmas.

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Conflict of Interest: All the authors declare that there is no conflict of interest in this study.

Author Contribution: EŞ was responsible for establishing and designing the surveys and experiments. EŞ and MEG conducted the experiments, wrote the manuscript and analysed the data. All authors have read and approved the manuscript.

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