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Molecular diversity of '*Candidatus* Phytoplasma' species in pome and stone fruits in Turkey

Türkiye'de yumuşak ve sert çekirdekli meyvelerde '*Candidatus* Phytoplasma' türlerinin moleküler çeşitliliği

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ABSTRACT

Apple, pear and apricot trees showing phytoplasma associated symptoms from Ankara and Isparta provinces were sampled and investigated to verify the presence of phytoplasma-associated diseases. Totally 31 samples were tested with phytoplasma universal and group specific primers and the PCR products were restricted using *Tru1*I, *Rsa*I and *Ssp*I endonucleases. Different RFLP profiles were obtained and selected samples were directly sequenced. The apple samples were found infected with 16SrX-A ('*Candidatus* Phytoplasma mali'), while the majority of the pear samples were infected with 16SrX-A and 16SrX-C subgroup phytoplasmas in mixed infection. The 16SrX-B ('*Candidatus* Phytoplasma prunorum'), 16SrX-C ('*Candidatus* Phytoplasma pyri') and mixed infection of 16SrX-A/16SrX-C and 16SrX-C/16SrI (aster yellows) were detected in the apricot samples. In this study the 16SrI phytoplasmas in apricot and the mixed phytoplasma infections in pear and apricot trees were detected in Turkey for the first time.

INTRODUCTION

Phytoplasmas are plant pathogenic, phloem-limited bacteria belonging to the class Mollicutes transmitted by psyllid vectors, vegetative propagation of infected plant material and seeds (Bertaccini and Lee 2018). Fruit trees are mostly affected by phytoplasmas in ribosomal group 16SrX, namely '*Candidatus* Phytoplasma mali' agent of the apple proliferation (AP) disease, '*Ca.* P. pyri' agent of the pear decline (PD) disease and '*Ca.* P. prunorum' associated with the European stone fruit yellows (ESFY) (Seemüller and Schneider 2004). In European Union, phytoplasmas are quarantine organisms and listed in the Annex I. Part A. Section II of the Council Directive 2000/29/EC/Annex I/A2 also in the quarantine list of Turkey (Anonymous 2018).

Turkey is one of the main genetic origins of many fruit tree varieties and rootstocks. Fruit production has a great importance for agriculture and trade of Turkey. There are many indigenous and local varieties of pome and stone fruits such as Arapkızı and Amasya apples, Deveci and Ankara pear, Şekerpare, Hacıhaliloğlu and Alkayısı apricots; some foreign varieties of apple and pear are also commonly cultivated. Phytoplasma-associated diseases and their insect

vectors have been studied in Turkey since the last decade. '*Ca*. P. prunorum' (Sertkaya et al. 2005), apple proliferation (Canik and Ertunç 2007) and pear decline (Sertkaya et al. 2005, Ulubaş Serçe et al. 2006) were reported in Turkey in stone fruits, apple and pear, respectively. The purposes of this study are to verify and differentiate the phytoplasmas present in economically important apple, pear and apricot cultivars using 16S rRNA gene for their identification and molecular comparison (Lee et al. 1998, Zhao et al. 2009).

MATERIALS AND METHODS

Leaf yellowing and upward curling symptoms were observed in apricot, severe leaf reddening and leaf curling in pear, leaf reddening, autumn blossom, rosette-like shoot development and enlarged stipules in apple. Samples were collected in both commercial and experimental orchards of Isparta and Ankara provinces (Figure 1). Apple and pear samples were collected in November, apricot samples were collected in June. Apple, pear and apricot varieties used in this study are listed in Table 2. Totally 31 samples (16 from apple, 10 from pear and 5 from apricot trees) were used for the molecular analysis. The samples hydrangea 8 classified in the 16SrI-B subgroup and aster yellows from apricot classified in the 16SrI-F (Bertaccini 2015) were used as positive controls.

Total DNA was extracted from the midribs of fresh plant tissue according to Prince et al. (1993). The DNA concentration was measured by spectrophotometer and all samples were amplified in direct PCR by universal phytoplasma detection primers P1 (Deng and Hiruki 1991) and P7 (Schneider et al. 1995). Nested PCRs with primers R16F2n (Gundersen and Lee 1992)/R16R2 (Lee et al. 1993) and R16mF2/16RmR1 (Gundersen and Lee 1996) were then performed on products of direct PCR diluted 1:30 with sterile distilled water (SDW). Phytoplasma group specific primer pairs were further employed in nested PCR (Table 1). PCR was performed with 1X $MgCl_2$ supplemented PCR buffer, 20 ng DNA template, 200 mM dNTP's, 10 pmol from each primer and 0.2 U Taq DNA polymerase (RedTaq, Sigma Aldrich). All PCR reactions were performed in a mixture of 25 µl and for each PCR reaction; one positive control and SDW as negative control were employed to check the reliability of the reaction. Amplification consisted of 35 cycles of the following steps: 1 min denaturation at 94 °C, annealing for 2 min at 50 °C, extension at 72 °C for 3 min and 72 °C for 10 min of final extension. Aliquot of 5 µl from were analysed by electrophoresis on 1% agarose gel and visualized by staining with ethidium bromide under UV transilluminator (312 nm).

The R16F2n/R16R2 and group specific primer pairs' amplicons were restricted using *Tru1*I, *Rsa*I and *Ssp*I (Fermentas Fast Digest, Lithuania) endonucleases for RFLP (Restriction Fragment Length Polymorphism) analysis. Depending on the PCR band intensity approximately 3-6 µl of amplicon (corresponding to 300 ng of DNA) were used. Fragments were analyzed on 6.7% polyacrylamide gel and visualized as described above.

Selected amplicons, showing different RFLP profiles and obtained with primers R16mF2/R1 and/or R16F2n/R2 were purified with a QIAquick PCR Purification Kit (QIAGEN, CA, USA) and directly sequenced using the same primers employed for the amplification. Phytoplasma DNA sequences were aligned and a consensus sequence was obtained. Phylogenetic trees were constructed using '*Candidatus* Phytoplasma' species and other phytoplasmas

Figure 1. Symptomatic apple (a and b) and pear (c) trees. Severe leaf reddening, enlarged stipules and bunchy rosette like shoot growth on apple, leaf deformation and reddening on pear

Table 1. Primer pairs for PCR reactions and restriction enzymes for RFLP analyses

Primer pairs	Reference	Amplicon length (bp)	Restriction endonucleases
P1/P7	Deng and Hiruki 1991, Schneider et al. 1995	1,800	RsaI SspI Tru1I
R16mF2/R1	Gundersen and Lee 1996	1,700	
R16F2n/R16R2		1,250	
R16(X)F1/R1	Lee et al. 1994	1,100	

obtained from the GenBank, *Acholeplasma laidlawii* was used as the outgroup to root the trees. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987); distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004). The analysis involved 53 nucleotide sequences, all positions containing gaps and missing data were eliminated. There were a total of 761 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013). Phylogenetic analysis of nucleotide sequences was evaluated through 1,000 bootstraps.

RESULTS

All the apple, pear and apricot plant samples tested and showing phytoplasma associated symptoms resulted positive in PCRs with both universal and group specific primers while the negative (asymptomatic) samples did not show amplification in both direct and nested PCR analyses. Nested amplification of the samples with R16F2n/R16R2 primer pair yielded about 1.2 kb products (Figure 2).

The phytoplasmas associated with the studied diseases were distinguished according to the restriction profiles of their amplicons. Some of the apple, pear and apricot samples showed different restriction profile from those expected.

The RFLP patterns of the samples were evaluated according to Lee et al. (1998). The samples from apple showed the expected RFLP profiles after digestion with *Ssp*I, *Rsa*I and *Tru1*I (Table 2 and data not shown) corresponding to those of phytoplasmas in 16SrX-A subgroup. The majority of the RFLP profiles of the pear samples did show the presence of mixed phytoplasma infection with *Ssp*I and *Rsa*I restriction enzymes since the total length of the obtained bands exceeded the one expected from the digested amplicons (Figure 3). The profiles showed mixed infection of 16SrX-A and 16SrX-C profiles (Table 2) indicating a mixed infection of '*Ca*. P. mali' and '*Ca*. P. pyri'.

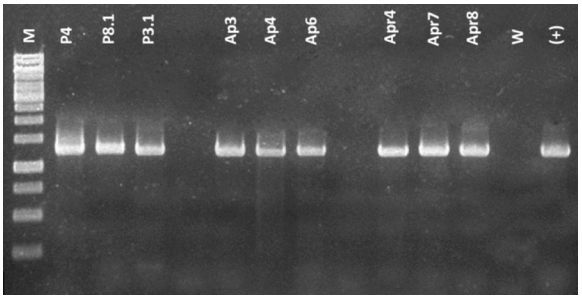

Figure 2. Agarose gel showing nested PCR amplicons obtained using R16F2n/R16R2 primer pair from pear, apple and apricot samples. Sample abbreviations are as reported in the Table 1; W: water; (+): hydrangea 8; M: 1 kb DNA ladder fragment sizes in base pairs from top to bottom of 10.000, 8.000, 6.000, 5.000, 4.000, 3.500, 3.000, 2.500, 2.000, 1.500, 1.000, 750, 500, 250

In the RFLP profiles of apricot samples the 16SrX-B phytoplasma profile was only detected in sample apricot 4, moreover in apricot 6, 7 and 8 the profiles obtained after *Tru1*I restriction did not correspond to those of the 16SrX phytoplasma group (apricot 6) or exceed the expected total length of 1,100 bp (apricot 7 and 8) indicating the presence of a possible mixed infection (Figure 4 and Table 2).

A total of six samples (two per species) were sequenced and the obtained aligned and manually revised sequences employed for phylogenetic analyses that confirmed the presence of 16SrX-A ('*Ca*. P. mali') phytoplasma in the two apple and in one pear samples, of the 16SrX-C ('*Ca*. P. pyri') phytoplasma in one pear and one apricot samples and finally of a 16SrI phytoplasma ('*Ca*. P. asteris', aster yellows) in one apricot sample (Figure 5). The obtained sequences were 99-100% identical to the respective reference strains and were submitted to the GenBank under numbers MK350301-MK350306 (Table 2).

Table 2. Results on phytoplasma detection in the apple, pear and apricot varieties (in bold the samples that were sequenced followed by the GenBank accession numbers)

Host Plant	Variety	Province	Strain	PCR results	RFLP phytoplasma identification / GenBank accession number
Apple	Grannysmith	Ankara	Ap1	positive	16SrX-A
	Grannysmith	Ankara	Ap2	negative	-
	Grannysmith	Ankara	Ap3	positive	16SrX-A
	Grannysmith	Ankara	Ap4	positive	16SrX-A
	Starkrimson	Isparta	Ap6	positive	16SrX-A
	Starkrimson	Isparta	Ap9	negative	-
	Starkrimson	Isparta	Ap16	positive	16SrX-A
	Starking	Ankara	ApB1	positive	16SrX-A
	Starking	Ankara	ApB2	positive	16SrX-A
	Starking	Ankara	ApB3	positive	16SrX-A / MK350301
	Starking	Ankara	ApB4	positive	16SrX-A / MK350302
	Starking	Ankara	ApB5	positive	16SrX-A
	Krimson	Isparta	ApE1	positive	16SrX-A
	Krimson	Isparta	ApE2	positive	16SrX-A
	Grannysmith	Isparta	ApE3	positive	16SrX-A
	Grannysmith	Isparta	ApE4	negative	-
Pear	Abbe Fetel	Isparta	P2	positive	16SrX-C + 16SrX-A
	Abbe Fetel	Isparta	P3	negative	-
	Santa Maria	Ankara	P4	positive	16SrX-C / MK350306
	Santa Maria	Ankara	P5	positive	16SrX-C + 16SrX-A
	Ankara	Ankara	D4	positive	16SrX-C + 16SrX-A
	Ankara	Ankara	TopA	positive	16SrX-C + 16SrX-A
	Ankara	Ankara	P8.1	positive	16SrX-C + 16SrX-A
	Ankara	Ankara	P3.1	positive	16SrX-C + 16SrX-A /
	Ankara	Ankara	P3.2	positive	MK350305
	Ankara	Ankara	P10.2	positive	16SrX-C + 16SrX-A
					16SrX-C + 16SrX-A
Apricot	Unknown	Isparta	Apr4	positive	16SrX-B
	Unknown	Isparta	Apr5	positive	16SrX-C
	Unknown	Isparta	Apr6	positive	16SrX-C + 16SrI / MK350303
	Unknown	Isparta	Apr7	positive	16SrX-A + 16SrX-C /
	Unknown	Isparta	Apr8	positive	MK350304
					16SrX-C

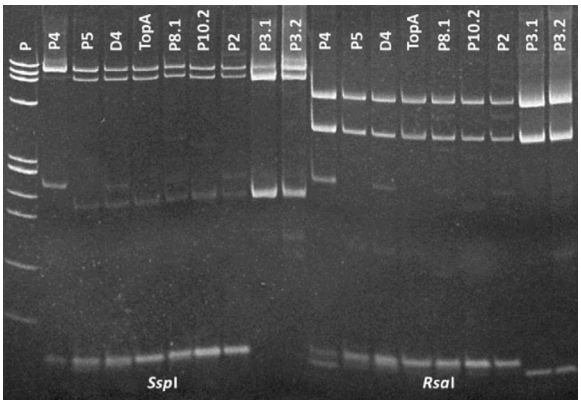

Figure 3. *Ssp*I and *Rsa*I profiles of R16(X)F1/R1 amplicons obtained from pear samples. Sample abbreviations are as reported in the Table 1; P, marker phiX174 *Hae*III digested with fragment sizes in base pairs from top to bottom of 1.353, 1.078, 872, 603, 310, 281, 271, 234, 194, 118 and 72

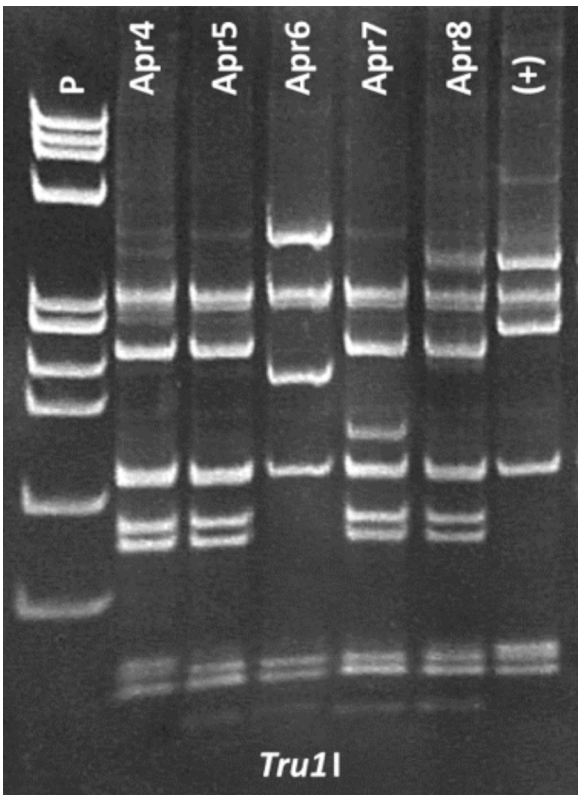

Figure 4. *Tru1*I profiles of R16F2n/R2 amplicons from apricot samples; sample abbreviations are as reported in the Table 1; (+): hydrangea 8; P, marker phiX174 *Hae*III digested with fragment sizes in base pairs from top to bottom of 1.353, 1.078, 872, 603, 310, 281, 271, 234, 194, 118 and 72

DISCUSSION

The detection of phytoplasmas in symptomatic fruit trees was obtained in this study by nested PCR with phytoplasma-specific primers and by RFLP and sequence analysis. This report is confirming the presence of the apple proliferation phytoplasma in apple first reported in Turkey in 2007 (Canik and Ertunç 2007), these infected trees were cut down because of the remarkable yield losses that reached about 65%. Grafting is the common vegetative propagation method for fruit tree that is also generating the transmission of the phloem restricted pathogens like phytoplasmas. There have not been any reported insect vector for the apple proliferation disease in Turkey, therefore, it can be speculated that the source of the AP may be the imported propagation material.

In the other two species studied the specific phytoplasmas (i.e '*Ca*. P. pyri' and *Ca*. P. prunorum') were detected in the majority of the cases in mixed infection with '*Ca*. P. mali' or '*Ca*. P. pyri'. Reports of the presence of both apple proliferation and pear decline phytoplasmas in diseased pear in Hungary (Del Serrone et al. 1998) and of apple proliferation phytoplasmas in cherry and other stone fruit trees in Slovenia (Mehle et al. 2007) indicate the possibility of cross infection of fruit tree phytoplasmas possibly related to the insect vectors feeding. *Cacopsylla pyri* and *C. pruni* were reported as insect vectors of PD and ESFY phytoplasmas respectively in Turkey (Ulubaş Serçe et al. 2006, 2011), respectively. The pome fruit and stone fruit orchards object of this study are next to each other and the source of the pear decline phytoplasma detected in apricot may the insect vector of ESFY (*C. pruni*). The presence of pear decline phytoplasmas in severely diseased peach orchards in Catalonia and Lleida regions of Spain (Garcia-Chapa et al. 2003, Lavina et al. 2015) and in Argentina (Fernandez et al. 2017) was reported and in these cases it could be linked to both insect vector transmission and infected propagation materials. The symptoms observed in apricot in Turkey resemble those described for peach in California (USA) (Blomquist and Kirkpatrick 2002) and Spain (Sabaté et al. 2014), also associated to the presence of `*Ca*. P. pyri´. Moreover the transmission of the pear decline phytoplasma to peach with *C. pyri* was demonstrated (Sabaté et al. 2018). There have not been any reports about the presence of '*Ca*. P. pyri' in apricot or in other stone fruits in Turkey. Gazel et al. (2009) reported that '*Ca*. P. prunorum' presence is associated with a yield loss of about 77% in some cases in the Mediterranean region. Ulubaş Serçe et al. (2007) examined the response of six apricot cultivars to '*Ca*. P. prunorum' and reported that at the end of the third year of the experiment the infected varieties died because of the infection. The presence of *C. pruni* in Turkey was found in Adana, Mersin,

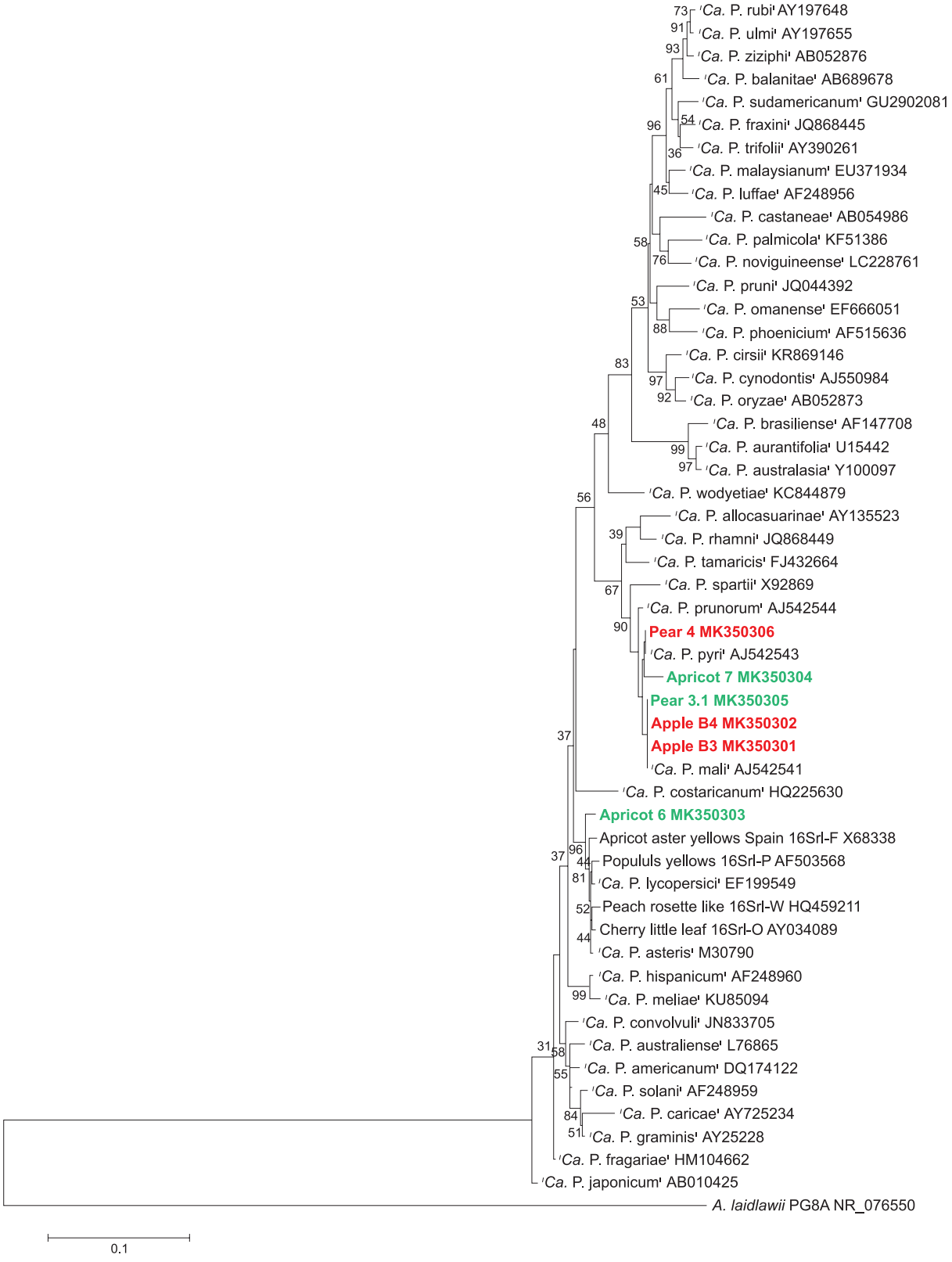

Figure 5. Evolutionary relationships of the fruit tree phytoplasmas from Turkey on 16S rDNA (red and green in the tree, sample abbreviations are as in the Table 1). The optimal tree obtained with the sum of branch length = 1.98333604 is presented. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Evolutionary analyses were conducted in MEGA6

Bursa and Yalova on either *Prunus spinosa* or wild *Prunus* spp., the mean of individuals of *C. pruni* infected with '*Ca*. P. prunorum' was around 23% in Mersin (Ulubaş Serçe et al. 2011). In pear, the yield loss is also remarkable. *Cacopsylla pyri* has been reported as the vector of '*Ca.* P. pyri' in the Mediterranean region (Sertkaya et al. 2008) and Bursa and Marmara region (Çağlayan et al. 2008). Also the detection of a 16SrI phytoplasma in apricot is not the first since there is a strain that was detected in apricot in Spain (Lee et al. 1998) and that resulted phylogenetically related to the one detected in this survey. On the other hand aster yellows phytoplasmas ('*Ca*. P. asteris') were reported in Turkey in cherry (Çağlayan et al. 2013) and in pomegranate (Gazel et al. 2016) and in peach in Canada (Zunnoon-Khan et al. 2010) indicating their spreading to fruit tree host plants.

This study revealed the existence of mix phytoplasma infection both in pome and stone fruit orchards in Turkey. For further verification of the relevance of mix phytoplasma infection on these and other fruit trees in Turkey larger surveys should be carried out. To the best of our knowledge, the 16SrI phytoplasma ('*Ca*. P. asteris', aster yellows) infection was detected for the first time in apricot in Turkey.

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ÖZET

Ankara ve Isparta illerinden fitoplazma enfeksiyonu benzeri belirti gösteren elma, armut ve kayısı ağaçlarından fitoplazma varlığının saptanmasına yönelik örnekleme yapılmıştır. Toplamda 31 örnek universal ve grup spesifik fitoplazma primerleri ile PCR'a tabi tutulmuş ve elde edilen PCR ürünleri *Tru1*I, *Rsa*I and *Ssp*I endonükleazları ile kesilmiştir. Elma örnekleri 16SrX-A ('*Candidatus* Phytoplasma mali') ile enfekteli bulunurken armut örneklerinin çoğunluğunda 16SrX-A ve 16SrX-C alt gruplarıyla karışık enfeksiyon saptanmıştır. Kayısı örneklerinde 16SrX-B ('*Candidatus* Phytoplasma prunorum'), 16SrX-C ('*Candidatus* Phytoplasma pyri') ve 16SrX-A/16SrX-C ve 16SrX-C/16SrI (aster yellows) alt gruplarının karışık enfeksiyonu saptanmıştır. Bu çalışma ile kayısıda 16SrI, armutta ve kayısıda karışık fitoplazma enfeksiyonları Türkiye'de ilk kez saptanmıştır.

Anahtar kelimeler: Fitoplazma tespiti, Yumuşak çekirdekliler, Kayısı, Karışık fitoplazma enfeksiyonu, Aster yellows grup

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