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**Multigene characterization of ‘*Candidatus Phytoplasma solani*’ associated
with a maize leaf reddening disease in Turkey**

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Abstract

In recent years, empty or poor grain bearing corn plants with leaf reddening symptoms have been observed in some commercial maize production areas in Adana province of Turkey. The disease has an ongoing increasing trend and cause economic losses. To verify the possible presence of phytoplasmas, leaf samples were tested with P1/Tint and R16F2n/R2 phytoplasma specific primer pairs after DNA extraction. The 16S ribosomal gene sequence analysis followed by sequence *in silico* enzyme digestions and phylogenetic analyses, allowed the identification of ‘*Candidatus Phytoplasma solani*’, subgroup of 16SrXII-A related-strain in all symptomatic samples. The positive samples were subjected to multigene analyses for the molecular characterization of *tuf* and *vmpl* genes. *In silico* restriction fragment length polymorphism and sequence analyses allowed the inclusion of all the positive samples into *tuf*-type b and V15 molecular types. The sequence analysis indicated the presence of a single genotype in all the tested samples (n=12). This is the first molecular characterization of a ‘*Ca. P. solani*’ strain infecting maize plants in Turkey. The identified pathogen is listed in the EPPO A2 list.

Key words: *Zea mays* L., phytoplasma, 16SrXII-A subgroup, PCR, *tuf*, *vmpI*

1 | INTRODUCTION

Maize (*Zea mays* L.) is a widely cultivated annual species in the *Poaceae* family followed by wheat and rice throughout the world. Maize is generally cultivated in humid regions in most parts of the world where 90% of its production is used for human and livestock feeding. It is grown in almost all regions of Turkey and has a great economic value to the national economy with a production of 6,500,000 tons. Adana province significantly contributes to the Turkish economy with the 12.6% of national maize production (819.978 tons) (Anonymous, 2020). Diseases associated with phytoplasmas in maize have been reported in some European countries including Serbia, Romania, Bulgaria, and more recently north Italy, Hungary, Bosnia and Herzegovina, Russia and India (Jović *et al.*, 2009; Calari *et al.*, 2010; Acs *et al.*, 2011; Kovačević *et al.*, 2014; Grigoryevna, 2015; Rao *et al.*, 2017). Maize redness, also known as corn reddening, is a severe disease associated with the presence of ‘*Ca. P. solani*’ or ‘*Ca. P. asteris*’ that belongs to the 16SrXII-A and 16SrI-B ribosomal subgroups, respectively (Quaglino *et al.*, 2013; Rao *et al.*, 2017). Maize redness disease is transmitted in Serbia by the cixiid planthoppers, *Reptalus panzeri* (Löw) and *Hyalesthes obsoletus* Signoret (Hemiptera: Cixiidae) (Jović *et al.*, 2007; 2009; Mori *et al.*, 2013). Duduk and Bertaccini (2006) reported the first presence of phytoplasmas in corn with reddening symptoms in Serbia, then, maize redness disease associated with the same phytoplasma was reported in Hungary and Bulgaria (Acs *et al.*, 2011; Genov *et al.*, 2014). ‘*Ca. P. solani*’ has a wide range of diverse host plants and many of them are of economic importance. Several studies have focused on its molecular identification and multilocus typing, moreover its genetic diversity was evaluated by different

protein coding-genes and molecular approaches (Cimerman *et al.*, 2009; Fialová *et al.*, 2009; Quaglino *et al.*, 2019). Based on *tuf* gene, ‘*Ca. P. solani*’ consists of two main strains named *tuf*-type a and b; while the *tuf*-type a strain is having an epidemic cycle that involves the stinging nettle (*Urtica dioica*), the *tuf*-type b is primarily associated with field bindweed (*Convolvulus arvensis*) (Langer and Maixner, 2004). The gene encoding variable membrane protein 1 (*vmp1*) has been employed for molecular characterization of immunodominant proteins linked to phytoplasma-host interactions (Cimerman *et al.*, 2009); it is more variable and has shown its usefulness for ‘*Ca. P. solani*’ epidemiological studies (Murolo *et al.*, 2010; Aryan *et al.*, 2014; Conigliaro *et al.*, 2020).

In Turkey, phytoplasma symptoms in maize plants have recently been detected and studied due to their increasing incidence in fields (Çağlar *et al.*, 2016, 2019). In the last two years, symptoms such as redness on leaf veins, empty and poor seed set in corn cobs were commonly observed in maize fields between April and May. This study was, therefore, designed to determine if these symptoms on maize plants are associated with ‘*Ca. P. solani*’ presence, and to verify the possible presence of genetic diversity based on *tuf* and *vmp1* genes in the detected phytoplasma strain(s).

2 | MATERIALS AND METHODS

2.1 | Sample collection

Leaf samples were collected from commercial maize fields expressing reddening symptoms in Adana province, Turkey and tested to verify possible phytoplasma presence and identity. Samples were collected in an area of two hectares, during surveys between April-May 2020. A total of 12 plants exhibiting the symptoms of reddening disease in the midrib of leaf and stem carrying empty corn cob with poor seed set were sampled (three per field) from four

neighboring fields (Figure 1). One asymptomatic plant sample from each field was also collected as a negative control.

2.2 | Nucleic acid extraction

DNA was extracted to determine the possible phytoplasma occurrence from midribs of fresh leaves following the method of Ahrens and Seemüller (1992) with some minor modifications. In brief, 1 g midrib was homogenized in 4 ml of CTAB buffer pH 8.0 (2% w/v cetyl trimethyl ammonium bromide, 1.4 mol L⁻¹ NaCl, 20 mmol L⁻¹ EDTA, 100 mmol L⁻¹ Tris HCl, 0.2% 2-β-mercaptoethanol, 2% polyvinylpyrrolidone-40) followed by an incubation at 65°C for 30 min. After elimination of plant tissue residues by centrifugation at 13,000 g for 10 min, the nucleic acids in suspension were clarified through chloroform–isoamyl alcohol (24:1), and precipitated with isopropanol at –20°C overnight. The precipitated nucleic acids were then collected by centrifugation at 13,000 g for 15 min, washed with 70% ethanol, dried at room temperature and finally dissolved in 50 µL of sterile double distilled water (SDDW). Before using in polymerase chain reaction (PCR) assays, nucleic acids were quantified and diluted with SDDW to a final concentration of 20 ng/µL.

2.3 | Detection and molecular characterization of phytoplasma DNA

The phytoplasma detection was carried out through nested PCR assays using the primer pair P1 (Deng and Hiruki, 1991) / Tint (Smart *et al.*, 1996) amplifying a fragment of 1,600 bp from the 16S rRNA gene and the beginning of the 16S-23S spacer region, followed by R16F2n/R16R2 primers (Gundersen and Lee, 1996), which amplify fragments of 1,250 bp in the 16S rRNA gene. All the samples amplified with R16F2n/R16R2 primer pair were used for amplification of *vmp1* and *tuf* genes using fTuf1/rTuf1 and StolH10F1/R1 primer pairs

followed by nested-PCR with fTufAY/rTufAY and TYPH10F/R primer pairs, respectively (Schneider *et al.*, 1997; Cimerman *et al.*, 2009; Fialová *et al.*, 2009). A DNA concentration of 40 ng was used as template in the final volume of 50 µL containing 1 µL dNTPs (10 mmol L⁻¹ mM), 1 µL of each primer (10 pmol), 5 µL of 10X Dream Taq green buffer, 0.25 µL Dream Taq DNA polymerase (5 U/µL) (Thermo Scientific, USA) and 40.75 µL SDDW. PCRs using P1/Tint, fTuf1/rTuf1 and H10F1/R1 were carried out after initial denaturation for 3 min at 95°C followed by 35 cycles of 1 min at 95°C, 1 min at 60°C (for P1/Tint) and 55°C (for fTuf1/rTuf1 and H10F1/R1) and 2 min at 72°C followed by an extension step of 10 min at 72°C in Mini Amp Plus Thermal Cycler (Applied Biosystems by Thermo Fisher Scientific). Nested-PCR assays using R16F2n/R16R2, fTufAY/rTufAY and TYPH10F1/R1 were performed using 1 µL of a 1: 50 dilution in SDDW of the PCR product. All the parameters were identical in direct and nested PCR except for the annealing temperatures, which were 55-, 50- and 53°C for the primers R16F2n/R16R2, fTufAY/rTufAY and TYPH10F1/R1, respectively. Samples without DNA template were added as negative controls in all PCR reactions. Total nucleic acid of the BGWL-Tu-M1 ('*Ca. P. cynodontis*', GenBank accession number HE599389) strain (Çağlar *et al.*, 2013) was employed as positive control. A strain of '*Ca. P. solani*' (Güldür *et al.*, 2018) was used as positive control in the PCR studies with *tuf* and *vmp1* genes. Seven microlitres of amplicons were electrophoresed in 1% of 1X TAE agarose gel, stained with ethidium bromide and photographed under a UV transilluminator.

The nested-PCR products obtained using R16F2n/R16R2 and fTufAY/rTufAY primers from symptomatic maize plants were analyzed by RFLP. Enzymatic digestions were carried out in a final volume of 30 µL including 2 µL Fast Digest Green Buffer, 10 µL amplicons and 1 U restriction enzyme *HpaII* (Thermo Scientific, Lithuania), according to the manufacturer's instruction at 37°C for 20 min. The digested products were separated in 1.5% of 1X TAE agarose gel, stained with ethidium bromide and photographed under a UV transilluminator.

2.4 | Sequencing and phylogenetic relationships

The R16F2n/R2 and TYPH10F1/R1 amplicons from four fields were purified using ExoSAP-IT® for PCR product clean-up (Thermo Fischer) according to the manufacturer's instructions. The purified products were quantified in a nanodrop spectrophotometer and sequenced in both directions with the same primers used for amplification in an automated sequencing (Macrogen, The Netherlands and Medsantek, Turkey). Sequence editing and assembly were carried out using MEGA 7 (Kumar *et al.*, 2016). The sequences obtained were compared with those of phytoplasmas using the Basic Local Alignment Search Tool (BLASTn) system of the National Center for Biotechnology Information (<https://www.ncbi.nlm.nih.gov/>). Partial sequences from the 16S rRNA gene corresponding to the R16F2n/R2 fragments were subjected to *in silico* RFLP analyses with the *iPhyClassifier* tool (Zhao *et al.*, 2009). In addition to 16S rDNA, the RFLP patterns of *vmpI* gene amplicons was studied to verify the phytoplasma identification accuracy. The nucleotide sequences were virtually digested with *RsaI* endonuclease via pDRAW32 software version 1.1.146 (AcaClone software, <http://www.acaclone.com>).

Phylogenetic trees were generated using MEGA 7 with the neighbor-joining method and 1,000 replicates for the bootstrap analysis to estimate the stability of the inferred subclades. The 16S rRNA gene sequence from *Acholeplasma laidlawii* (GenBank accession number M23932) was used as an outgroup for the 16S rDNA tree root. Twenty-one nucleotide sequences shown in Table 1 were downloaded from the GenBank database and used together with the nucleotide sequences generated in this study to reconstruct the phylogenetic relationships according to *vmpI* gene.

3 | RESULTS

The symptomatic maize plants, mainly located at the edge of the surveyed fields, exhibited severe reddening symptoms in leaves and midribs, and showed atrophied inflorescence and empty corn cob with poor quality of seeds (Figure 1). The use of P1/Tint primer pair enabled to amplify DNA bands (about 1.6 kb) from the positive control and from all symptomatic leaf tissues. However, no bands were observed from the asymptomatic plants negative control (data not shown). The 16S rDNA partial sequences of samples Tr-AcM 1, -2, -3 and -4 showed 100% identity with each other and 99.75% with that of '*Ca. P. solani*' (GenBank accession number AF248959) and were submitted to GenBank under the accession numbers MW344059, MW344060, MW344061, MW344062, respectively. The identification was made with *iPhyClassifier* based on *in silico* RFLP patterns (similarity coefficient 1.00) and this confirmed that the detected phytoplasma belong to the 16SrXII-A subgroup (Figure 2). This identification was further supported by the clustering analysis with other '*Candidatus* Phytoplasma' species and '*Ca. P. solani*' strains deposited to GenBank from Turkey and neighboring countries (Figure 3).

For the *tuf* and *vmpI* genes, amplification of approximately 1.4 kb and 0.9 kb, was obtained from the nested-PCR assays with TYPH10F1/R1 and fTufAY/rTufAY primer pairs, respectively (data not shown). Restriction patterns of PCR products from the *tuf* gene with *HpaII* were identical to each other and confirmed that all maize strains belong to *tuf*-type-b (Figure 4). The primer pair TYPH10F1/R1 also amplified the expected length fragment in all the maize samples and positive control but not from the negative controls. The *vmpI* amplicons of Tr-AcM 1, -2, -3 and -4 samples were sequenced in both directions, and the sequences were deposited in GenBank under the accession numbers MW984610, MW984611, MW984612, MW984613, respectively. The unrooted phylogenetic tree (Figure 5) constructed using the *vmpI* sequences listed in Table 1 and those obtained from corn from Turkey showed that the nucleotide sequences of maize strains clustered according to the typology of the *RsaI*/RFLP

(Figure 6). *Vmpl* gene amplicons of the four studied strains were virtually digested using the pDraw32 software that showed only one *RsaI* profile, identical to the one of the ‘*Ca. P. solani*’ strains enclosed in the lineage V15 (Figure 6).

4 | DISCUSSION

In this study, the presence of ‘*Ca. P. solani*’ in commercial maize production areas in Southern Anatolia including Adana province, was reported for the first time in Turkey. While the phytoplasmas in the 16SrXII group are divided in numerous subgroups distributed in agricultural crops worldwide, only subgroup 16SrXII-A was detected, indicating the presence of a homogeneous phytoplasma population. A prerequisite to manage newly occurring diseases is the detection and proper identification of the associated pathogen. The identification of this phytoplasma strain in corn is, therefore, a first step for the management of the reddening of maize disease via cultural, chemical and breeding approaches.

The molecular characterization of ‘*Ca. P. solani*’ in maize by using the *tuf* and *vmpI* genes confirmed the finding and the phytoplasma identity and represents a new epidemiological information about ‘*Ca. P. solani*’ in Turkey. Based on the *tuf* gene characterization, all the symptomatic maize samples showed the presence of only *tuf*-type b which agrees with reports from Bosnia and Herzegovina (Kovačević *et al.* 2014; Delić *et al.*, 2016). *Tuf*-type b is primarily associated with field bindweed (*Convolvulus arvensis* L.) (Langer and Maixner, 2004) that is its dominant reservoir plant species throughout most of the Europe and Turkey (Ember *et al.*, 2011; Ozturk *et al.*, 2017). With the abundance of these plants, ‘*Ca. P. solani*’ has a strong potential to spread out to maize and other crop plants when the insect vector(s) are present. Typing *vmpI* gene with *RsaI* digestion and sequence analysis indicated that all maize strains in the present study belonged to the same molecular type named V15. It was previously identified in the 22% of 56 *Hyalestes obsoletus* in Spanish vineyards (Sabaté *et al.*, 2014), in

199 diseased grapevine and *Convolvulus arvensis* (Murolo *et al.*, 2010; Sabaté *et al.*, 2014;
200 Quaglino *et al.*, 2016; Jamshidi *et al.*, 2019), and in tomato (Contaldo *et al.*, 2021) in other
201 Mediterranean Countries. In the present study, all symptomatic maize samples resulted infected
202 by this phytoplasma strain. Identification of the same genetic variant of ‘*Ca. P. solani*’ from
203 different fields in the present study indicated that the pathogen population is strongly
204 homogeneous, and the pathogens have a probable common origin. A similar case with severe
205 corn reddening symptoms associated with a similar strain of phytoplasma was reported in
206 Bulgaria (Genov *et al.*, 2014) considering that close geographical areas could create potential
207 danger for insect vector mobility. Limited studies have reported the presence of *H. obsoletus*
208 and *Dictyophara europaea*, insect vectors of 16SrXII-A phytoplasmas, in vineyards of Turkey.
209 However, the phytoplasma presence in those insect species were negatively reported previously
210 (Bayram *et al.*, 2014). Moreover, *Laodelphax striatellus* (Fallen, 1826), a possible vector of
211 16SrI, 16SrXII-A and 16SrIII group phytoplasmas (Prota *et al.*, 2007), was previously detected
212 in Turkish maize fields (Sertkaya *et al.*, 2010; Bayram *et al.*, 2014). Çukurova region is a large,
213 flat and fertile area suitable for agricultural crop production where almost all agricultural
214 products except tea and hazelnuts could be grown. Since this region is suitable for
215 polycultivation due to the suitable climatic conditions and long vegetation period, two or more
216 harvests can be possible for different crops in one season. As a result of this, any occurrence of
217 new disease or emerging pathogen has great potential in terms of spread mainly due to insect
218 vectors . It is known that phytoplasmas are spread primarily by insects families such as
219 *Cicadellidae* (leafhoppers), *Fulgoridae* (planthoppers), and *Psyllidae* (jumping plant lice),
220 which feed in the phloem of plants, and, in case of infected plants, they may transmit them to
221 the next plant on which they feed. Thus, the host range of phytoplasmas strongly depends on
222 the insect vector (Weintraub and Beanland, 2006). Inthe reported case, it is suggested that insect
223 vectors might have played a significant role for the spread of phytoplasmas to maize plants.

Therefore, the cultivation of maize varieties resistant to the diseases associated with phytoplasmas and the control of insect vectors may be of great significance to manage this emerging disease situation. Further studies are, however, necessary to identify the potential insect vectors, the origin of the disease and its geographical distribution to develop a sustainable disease management. With the global increase in temperature, xerothermic insect vectors might migrate towards northern areas, and the cultivation of various host plant species in the same area would probably increase the possibility of phytoplasma dissemination (Maixner and Langer, 2006; Krishnareddy, 2013; Mamay and Şimşek, 2017). The phytoplasma-associated disease emergence in new geographic areas might create epidemics where susceptible host plant species are cultivated. Therefore, the main strategy for the control of the phytoplasma diseases is to monitor their presence and consider the biological activities of the insect vectors.

CONFLICT OF INTEREST

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

AUTHOR CONTRIBUTION

BKÇ was responsible for establishing and designing the surveys and experiments. BKÇ conducted the Nested-PCR experiments based on 16S rDNA, BKÇ, EŞ and MD conducted the experiments based on *Tuf* and *VMP1*. AB conducted phylogenetic studies. BKÇ, EŞ, AB and MD wrote the manuscript and analysed the data. All authors have read and approved the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure 1. Disease symptoms associated with the presence of ‘*Ca. P. solani*’ in maize. Top: maize reddening symptoms with red streak on vein of leaf and stem; bottom: empty corn cob and poor seed set formation from symptomatic maize plants.

Figure 2. Virtual RFLP patterns derived from *in silico* digestions of Tr-AcM-1, Tr-AcM-2, Tr-AcM-3 and Tr-AcM-4 strain sequences obtained from R16F2n/16R2 products (about 1.2 kb) and phytoplasma 16S rDNA gene fragments from strains enclosed in the 16SrXII subgroups with four key enzymes (*AluI*, *MseI*, *BstUI* and *HhaI*). Red boxes show the identity in the profiles of the strains (*AluI* and *MseI*), green boxes show the difference of the strains using *BstUI* and *HhaI*. MW: Marker, Φ X174 DNA profile digested with *HaeIII* with the following fragments in base pairs from top to bottom: 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118; 72.

Figure 3. Phylogenetic tree constructed by neighbor-joining method inferred from the ‘*Candidatus* Phytoplasma’ species nucleotide sequences of the 16S rDNA gene. The tree shows the relationships among the phytoplasma strains obtained from maize plants from Turkey (red squares) and some ‘*Candidatus* Phytoplasma’ species available in GenBank. The tree was constructed using MEGA 7 software. GenBank accession number and ribosomal subgroups are reported on the right of the strain names. *A. laidlawii* was employed as outgroup to root the tree.

Figure 4. RFLP analysis of the fTufAY/rTufAY amplicons of the elongation factor Tu (*tuf*) gene with *HpaII*. Only ‘*Ca. P. solani*’ *tuf*-type b was detected. M, marker 1 kb DNA ladder; +, Con: positive control from 16SrXII-A subgroup; UdC, undigested amplified control.

Figure 5. Unrooted phylogenetic tree inferred from the ‘*Ca. P. solani*’ strain nucleotide sequences of the *vmpI* gene. Minimum evolution analysis was carried out using the neighbor-joining method and bootstrap replicated 1,000 times. The full names of the phytoplasma strains included in the phylogenetic analysis are given in the Table 1. The GenBank accession number of each sequence is given in parenthesis; gene sequences obtained in the present study are indicated with red squares. Square brackets show the grouping obtained by digestion of the amplicons with the endonuclease *RsaI*.

Figure 6. Virtual RFLP patterns of *vmpI* gene sequence amplified with the primer pair TYPH10F/R of strains Tr-AcM1, -2, -3 and -4 with the P7 strain from periwinkle from Lebano as control (GenBank

416 accession number AM992100). M: marker, Φ X174 DNA profile digested with *Hae*III with the
417 following fragments in base pairs from top to bottom: 1,353; 1,078; 872; 603; 310; 281; 271;
418 234; 194; 118; 72.

419

420 **Table 1.** '*Ca. P. solani*' *vmpI* gene sequences available in GenBank used in the phylogenetic analysis.

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