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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 the Adana province of Turkey. The disease is increasing and causing 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 characterized using *tuf* and *vmp1* 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.

KEYWORDS

16SrXII-A subgroup, PCR, phytoplasma, *tuf*, *vmp1*, *Zea mays* L.

1 | INTRODUCTION

Maize (*Zea mays* L.) is a widely cultivated annual plant 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 consumption and livestock feeding. It is grown in almost all regions of Turkey with an annual 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 in north Italy, Hungary, Bosnia and Herzegovina, Russia and India (Acs et al., 2011; Calari et al., 2010; Grigoryevna, 2015; Jović et al., 2009; Kovačević et al., 2014; 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 host range with many hosts of high economic importance. Several studies have focussed on its molecular identification and multilocus typing of the pathogen, and its genetic diversity has been characterized by different protein coding-genes and molecular approaches (Cimerman et al., 2009; Fialová et al., 2009; Quaglino et al., 2019). '*Ca.* P. solani' consists of two main strains named tuf-type a and b; while the tuf-type strain has an epidemic cycle on the stinging nettle (*Urtica dioica*), the tuf-type b is primarily associated with field bindweed (*Convolvulus arvensis*) (Langer & 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

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variable and has shown its usefulness for '*Ca*. P. solani' epidemiological studies (Aryan et al., 2014; Conigliaro et al., 2020; Murolo et al., 2010).

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 whether these symptoms on maize plants caused by '*Ca*. P. solani', and to verify the potential genetic diversity based on the *tuf* and *vmp1* genes in the phytoplasma strain(s).

2 | MATERIALS AND METHODS

2.1 | Sample collection

Leaf samples exhibiting reddening symptoms were collected from several commercial maize fields in Adana province, Turkey, and tested to verify possible phytoplasma presence. Samples were collected in an area of two hectares, during surveys between April and May 2020. A total of 12 plants exhibiting symptoms of reddening disease in the midrib of leaf and stem carrying corn cobs with poor seed set were sampled (three per field) from four neighbouring 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 from the 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 NaCl, 20 mmol/L EDTA, 100 mmol/L Tris HCl, 0.2% 2-β-mercaptoethanol and 2% polyvinylpyrrolidone-40) followed by incubation at 65°C for 30 min. After the aggregation of plant tissue by centrifugation at 18,360 *g* for 10 min, the nucleic acids in the supernatant 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 18,360 *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

Nested-PCR assays using the primer pair P1 (Deng & 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 & Lee, 1996), which amplify fragments of 1,250 bp in the 16S rRNA gene, were employed to detect Phytoplasma. 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 (Cimerman et al., 2009; Fialová et al., 2009; Schneider & Gibb, 1997). A DNA concentration of 40 ng was used as template in the final volume of 50 µl containing 1 µl dNTPs (10 mmol/L 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 analysed 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 *Hpa*II (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



FIGURE 1 Symptoms associated with the presence of '*Candidatus* Phytoplasma solani' in maize. Top: maize reddening symptoms with red streak on foliar veins and stem; bottom: empty corn cob and poor seed set from symptomatic maize plants [Colour figure can be viewed at wileyonlinelibrary.com]

sequences from the 16S rRNA gene corresponding to the R16F2n/R2 fragments were subjected to in silico RFLP analyses with the *i*PhyClassifier tool (Zhao et al., 2009). In addition to 16S rDNA, the RFLP patterns of *vmp1* gene amplicons were studied to verify the phytoplasma identification accuracy. The nucleotide sequences were virtually digested with *Rsa*I endonuclease via pDRAW32 software version 1.1.146 (AcaClone software, http://www.acaclone.com).

Phylogenetic trees were generated using MEGA 7 with the neighbour-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 *vmp1* gene.

3 | RESULTS

The symptomatic maize plants, mainly located at the edge of the surveyed fields, exhibited severe reddening 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 (approximately 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 and MW344062, respectively. The identification was made with *i*PhyClassifier based on in silico RFLP patterns (similarity coefficient 1.00) and this confirmed that the detected phytoplasma belongs

TABLE 1 '*Candidatus* Phytoplasma solani' *vmp1* gene sequences available in GenBank used in the phylogenetic analysis

Strain	vmp1/*Rsa*I profile	Host	Country	Accession number
Rkat47	V1	*Vitis vinifera*	Georgia	KT184874
Char7	V1	*Vitis vinifera*	Georgia	KT184869
T2_92	V2	*Vitis vinifera*	Germany	AM992106
T2_56	V2	*Lycopersicum* sp.	Italy	AM992104
CH−1	V2	*Vitis vinifera*	Italy	AM992105
GGY	V2	*Vitis vinifera*	Germany	AM992102
Mri10	V3	*Vitis vinifera*	Italy	HM008615
Aa25	V3	*Vitis vinifera*	Italy	HM008614
N3	V3	*Urtica dioica*	Slovenia	JQ977723
Moliere	V4	*Prunus mahaleb*	France	AM992096
Charente1	V4	*Hyalesthes obsoletus*	France	AM992098
19–25	V5	*Vitis vinifera*	Germany	AM992101
Mag1	V5	*Vitis vinifera*	Italy	HM008613
Mvercer2	V12	*Vitis vinifera*	Italy	HM008612
400–05	V12	*Vitis vinifera*	Italy	EF655660
Ag4a	V14	*Vitis vinifera*	Italy	HM008605
C3	V14	*Vitis vinifera*	Italy	HM008603
Char8	V14	*Vitis vinifera*	Georgia	KT184870
P7	V15	*Catharanthus roseus*	Lebanon	AM992100
B7	V9	*Vitis vinifera*	Italy	HM008608
Mp49	V9	*Vitis vinifera*	Italy	HM008607

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 from Turkey and neighbouring countries in the GenBank (Figure 3).

For the *tuf* and *vmp1* 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 *Hpa*II 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 *vmp1* 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 and MW984613, respectively. The unrooted phylogenetic tree (Figure 5) constructed using the *vmp1* 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 *Rsa*I/RFLP (Figure 6). *Vmp1* gene amplicons of the four strains studied were virtually digested using the pDraw32 software that showed only one *Rsa*I 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 into 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 *vmp1* 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 (Delić et al., 2016; Kovačević et al., 2014). Tuf-type b is primarily associated with field bindweed (*Convolvulus arvensis* L.) (Langer & Maixner, 2004) that is its dominant reservoir plant species throughout most of Europe and Turkey (Ember et al., 2011; Ozturk et al., 2017). With the abundance of these plants, '*Ca*. P. solani' has

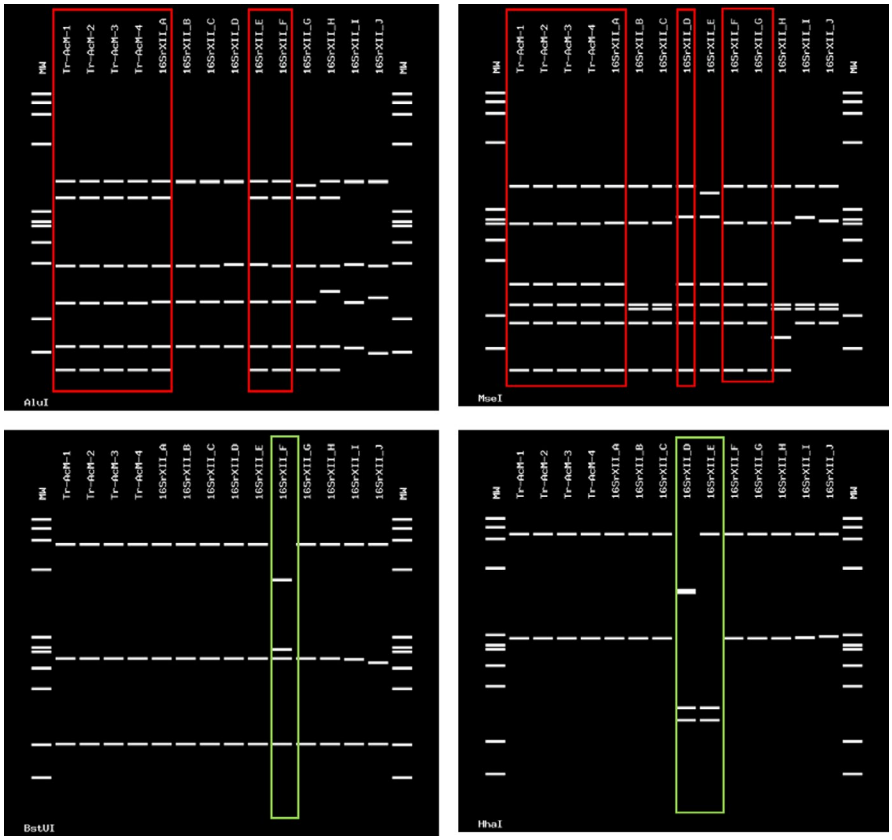


FIGURE 2 Virtual RFLP patterns derived from in silico digestions of Tr-AcM-1, Tr-AcM-2, Tr-AcM-3 and Tr-AcM-4 strain sequences obtained from R16F2n/16R2 products (approximately 1.2 kb) and phytoplasma 16S rDNA gene fragments from strains enclosed in the 16SrXII subgroups with four key enzymes (*Alu*I, *Mse*I, *Bst*UI and *Hha*I). Red boxes show the identity in the profiles of the strains (*Alu*I and *Mse*I), green boxes show the difference of the strains using *Bst*UI and *Hha*I. MW: Marker, ΦX174 DNA profile digested with *Hae*III with the following fragments in base pairs from top to bottom: 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118; 72 [Colour figure can be viewed at wileyonlinelibrary.com]

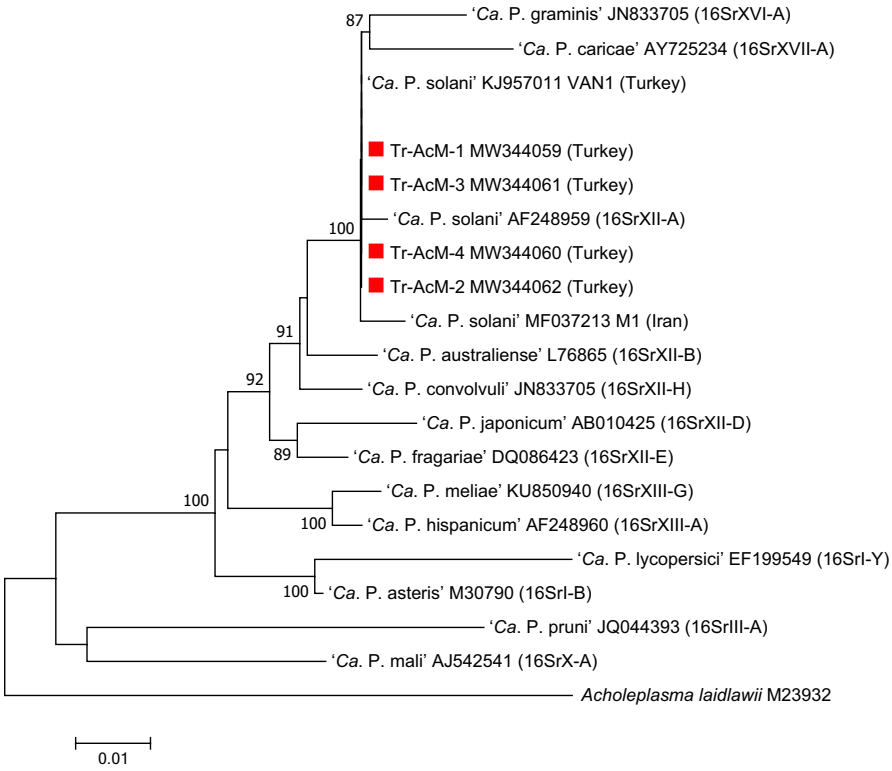


FIGURE 3 Phylogenetic tree constructed by neighbour-joining method inferred from the '*Candidatus* Phytoplasma' species nucleotide sequences of the 16S rDNA gene. The tree shows the relationships among the phytoplasma strains obtained from maize plants from Turkey (red squares) and some '*Candidatus* 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. *Acholeplasma laidlawii* was employed as outgroup to root the tree [Colour figure can be viewed at wileyonlinelibrary.com]

a strong potential to spread out to maize and other crop plants when the insect vector(s) are present. Typing *vmp1* gene with *Rsa*I 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 diseased grapevine

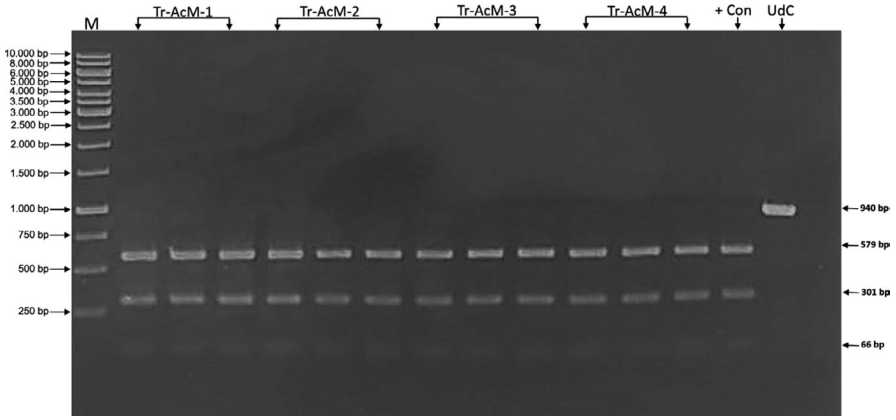


FIGURE 4 RFLP analysis of the fTufAY/rTufAY amplicons of the elongation factor Tu (tuf) gene with *Hpa*II. Only '*Candidatus* Phytoplasma solani' tuf-type b was detected. M, marker 1 kb DNA ladder; +, Con: positive control from 16SrXII-A subgroup; UdC, undigested amplified control

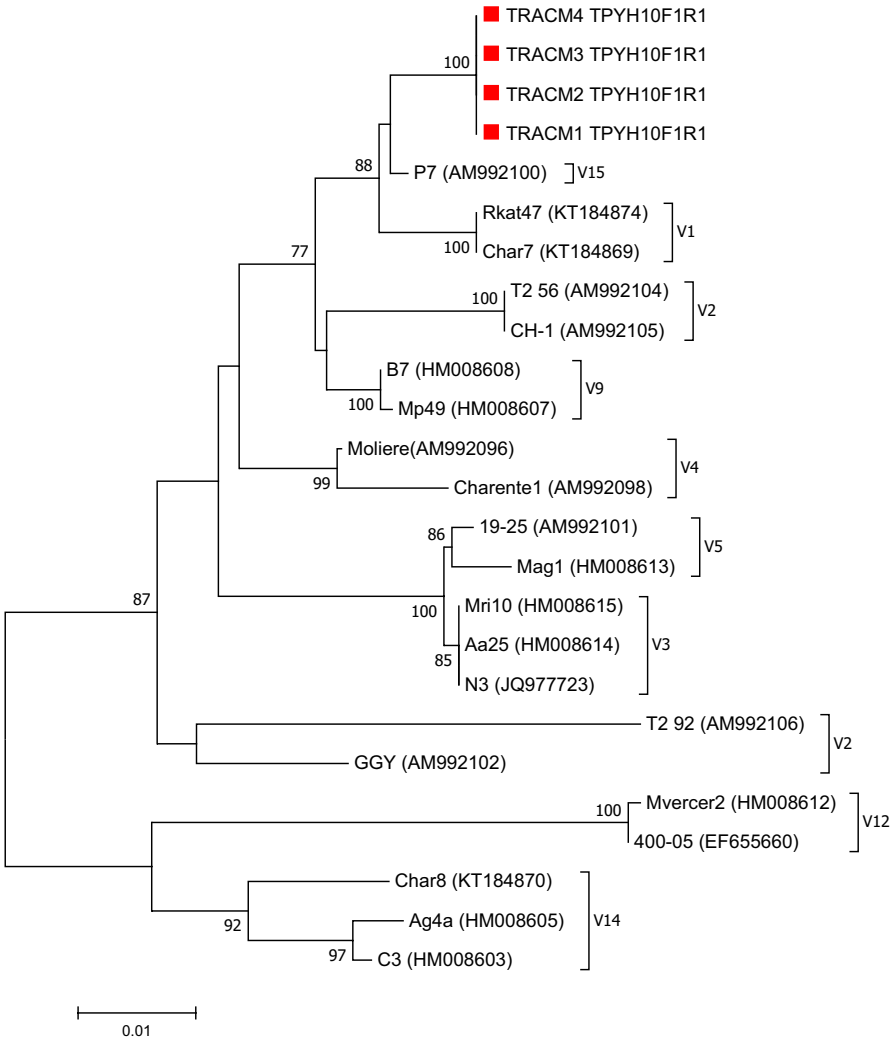


FIGURE 5 Unrooted phylogenetic tree inferred from the '*Candidatus* Phytoplasma solani' strain nucleotide sequences of the *vmp1* gene. Minimum evolution analysis was carried out using the neighbour-joining method and bootstrap replicated 1,000 times. The full names of the phytoplasma strains included 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 *Rsa*I [Colour figure can be viewed at wileyonlinelibrary.com]

and *Convolvulus arvensis* (Jamshidi et al., 2019; Murolo et al., 2010; Quaglino et al., 2016; Sabaté et al., 2014), and in tomato (Contaldo et al., 2021) in other Mediterranean countries. In the present study, all symptomatic maize samples were infected by this phytoplasma strain. Identification of the same genetic variant of '*Ca.* P. solani' from different fields suggested that the pathogen population is strongly homogeneous, and the strains have a probable common origin. A similar case with severe corn reddening symptoms associated with a similar strain of phytoplasma was reported in Bulgaria (Genov et al., 2014) considering that close geographical areas could create potential danger for insect vector mobility. Limited studies have reported the presence of *H. obsoletus* and *Dictyophara europaea*, insect vectors of 16SrXII-A phytoplasmas, in vineyards of Turkey. However, the phytoplasma presence in those insect species was reported as negative previously (Bayram et al., 2014). Moreover, *Laodelphax striatellus* (Fallen, 1826), a possible vector of 16SrI, 16SrXII-A and 16SrIII group phytoplasmas (Prota et al., 2007), was previously detected in Turkish maize fields (Bayram et al., 2014; Sertkaya et al., 2010).

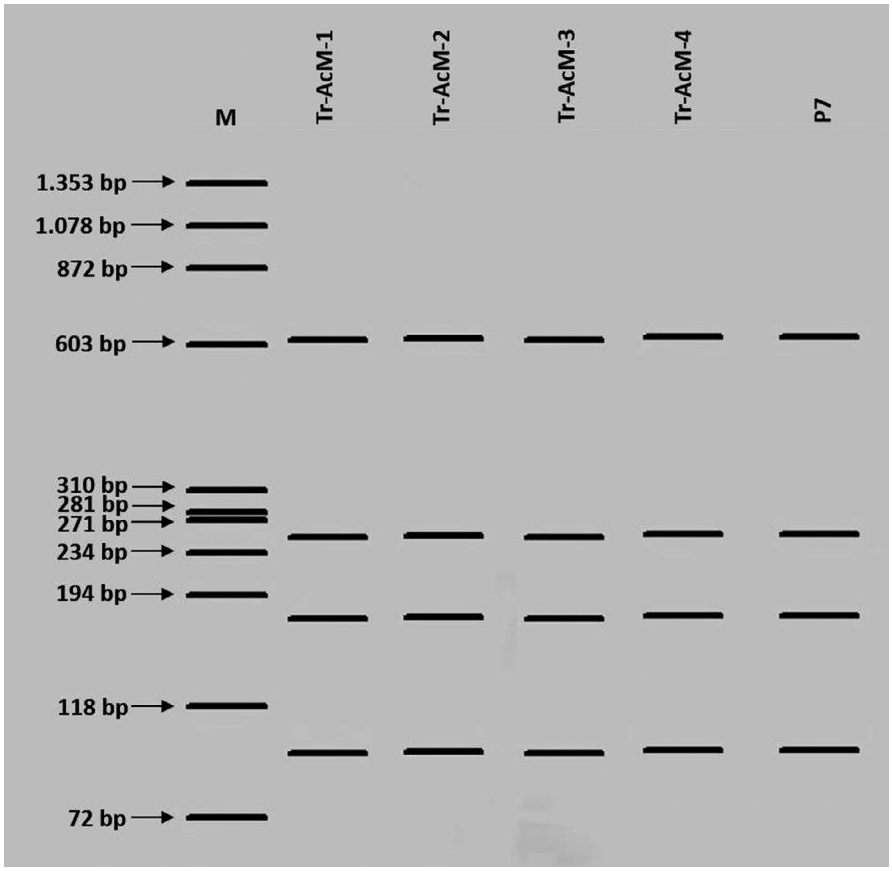


FIGURE 6 Virtual RFLP patterns of *vmp1* gene sequence amplified with the primer pair TYPH10F/R of strains Tr-AcM1, −2, −3 and −4 with the P7 strain from periwinkle from Lebano as control (GenBank accession number AM992100). M: marker, ΦX174 DNA profile digested with *Hae*III with the following fragments in base pairs from top to bottom: 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118; 72

Çukurova region is a large, flat and fertile area suitable for crop production where almost all agricultural products except tea and hazelnuts could be grown. Since this region is suitable for polycultivation due to the suitable climatic conditions and long vegetation period, two or more harvests can be possible for different crops in one season. As a result, any occurrence of new disease or emerging pathogen has great potential in terms of spread mainly due to insect vectors. It is known that phytoplasmas are spread primarily by insects families such as *Cicadellidae* (leafhoppers), *Fulgoridae* (planthoppers) and *Psyllidae* (jumping plant lice), which feed in the phloem of plants, and, in case of infected plants, they may transmit them to the next plant on which they feed. Thus, the host range of phytoplasmas strongly depends on the insect vector (Weintraub & Beanland, 2006). This study suggests that insect 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 is pivotal to manage this emerging disease. Further studies, however, are 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 (Krishnareddy, 2013; Maixner & Langer, 2006; Mamay & Şimşek, 2017). The phytoplasma-associated disease emergence in new geographic areas might create epidemics where susceptible host 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 CONTRIBUTIONS

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.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/jph.13036.

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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