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This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

Published Version:

Caglar B.K., Simsek E., Dikilitas M., Bertaccini A. (2021). Characterization of 'Candidatus Phytoplasma solani' associated with a maize leaf reddening disease in Turkey. JOURNAL OF PHYTOPATHOLOGY, 169(11-12), 658-666 [10.1111/jph.13036].

Availability:

This version is available at: <https://hdl.handle.net/11585/846693> since: 2022-01-21

Published:

DOI: <http://doi.org/10.1111/jph.13036>

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JOURNAL OF PHYTOPATHOLOGY Volume 169, Issue 11-12 December 2021 Pages 658-666

which has been published in final form at <https://dx.doi.org/10.1111/jph.13036>

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

3

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11

12 **Abstract**

13 In recent years, empty or poor grain bearing corn plants with leaf reddening symptoms have
14 been observed in some commercial maize production areas in Adana province of Turkey. The
15 disease has an ongoing increasing trend and cause economic losses. To verify the possible
16 presence of phytoplasmas, leaf samples were tested with P1/Tint and R16F2n/R2 phytoplasma
17 specific primer pairs after DNA extraction. The 16S ribosomal gene sequence analysis followed
18 by sequence *in silico* enzyme digestions and phylogenetic analyses, allowed the identification
19 of ‘*Candidatus Phytoplasma solani*’, subgroup of 16SrXII-A related-strain in all symptomatic
20 samples. The positive samples were subjected to multigene analyses for the molecular
21 characterization of *tuf* and *vmp1* genes. *In silico* restriction fragment length polymorphism and
22 sequence analyses allowed the inclusion of all the positive samples into *tuf*-type b and V15
23 molecular types. The sequence analysis indicated the presence of a single genotype in all the
24 tested samples (n=12). This is the first molecular characterization of a ‘*Ca. P. solani*’ strain
25 infecting maize plants in Turkey. The identified pathogen is listed in the EPPO A2 list.

26

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

28

29

30 1 | INTRODUCTION

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

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

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

67

68 **2 | MATERIALS AND METHODS**

69 **2.1 | Sample collection**

70 Leaf samples were collected from commercial maize fields expressing reddening
71 symptoms in Adana province, Turkey and tested to verify possible phytoplasma presence and
72 identity. Samples were collected in an area of two hectares, during surveys between April-May
73 2020. A total of 12 plants exhibiting the symptoms of reddening disease in the midrib of leaf
74 and stem carrying empty corn cob with poor seed set were sampled (three per field) from four

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

77

78 **2.2 | Nucleic acid extraction**

79 DNA was extracted to determine the possible phytoplasma occurrence from midribs of
80 fresh leaves following the method of Ahrens and Seemüller (1992) with some minor
81 modifications. In brief, 1 g midrib was homogenized in 4 ml of CTAB buffer pH 8.0 (2% w/v
82 cetyl trimethyl ammonium bromide, 1.4 mol L⁻¹ NaCl, 20 mmol L⁻¹ EDTA, 100 mmol L⁻¹ Tris
83 HCl, 0.2% 2-β-mercaptoethanol, 2% polyvinylpyrrolidone-40) followed by an incubation at
84 65°C for 30 min. After elimination of plant tissue residues by centrifugation at 13,000 g for 10
85 min, the nucleic acids in suspension were clarified through chloroform–isoamyl alcohol (24:1),
86 and precipitated with isopropanol at –20°C overnight. The precipitated nucleic acids were then
87 collected by centrifugation at 13,000 g for 15 min, washed with 70% ethanol, dried at room
88 temperature and finally dissolved in 50 µL of sterile double distilled water (SDDW). Before
89 using in polymerase chain reaction (PCR) assays, nucleic acids were quantified and diluted with
90 SDDW to a final concentration of 20 ng/µL.

91

92 **2.3 | Detection and molecular characterization of phytoplasma DNA**

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

99 followed by nested-PCR with fTufAY/rTufAY and TYPH10F/R primer pairs, respectively
100 (Schneider *et al.*, 1997; Cimerman *et al.*, 2009; Fialová *et al.*, 2009). A DNA concentration of
101 40 ng was used as template in the final volume of 50 μ L containing 1 μ L dNTPs (10 mmol L⁻¹
102 mM), 1 μ L of each primer (10 pmol), 5 μ L of 10X Dream Taq green buffer, 0.25 μ L Dream
103 Taq DNA polymerase (5 U/ μ L) (Thermo Scientific, USA) and 40.75 μ L SDDW. PCRs using
104 P1/Tint, fTuf1/rTuf1 and H10F1/R1 were carried out after initial denaturation for 3 min at 95°C
105 followed by 35 cycles of 1 min at 95°C, 1 min at 60°C (for P1/Tint) and 55°C (for fTuf1/rTuf1
106 and H10F1/R1) and 2 min at 72°C followed by an extension step of 10 min at 72°C in Mini
107 Amp Plus Thermal Cycler (Applied Biosystems by Thermo Fisher Scientific). Nested-PCR
108 assays using R16F2n/R16R2, fTufAY/rTufAY and TYPH10F1/R1 were performed using 1 μ L
109 of a 1: 50 dilution in SDDW of the PCR product. All the parameters were identical in direct
110 and nested PCR except for the annealing temperatures, which were 55-, 50- and 53°C for the
111 primers R16F2n/R16R2, fTufAY/rTufAY and TYPH10F1/R1, respectively. Samples without
112 DNA template were added as negative controls in all PCR reactions. Total nucleic acid of the
113 BGWL-Tu-M1 ('*Ca. P. cynodontis*', GenBank accession number HE599389) strain (Çağlar *et*
114 *al.*, 2013) was employed as positive control. A strain of '*Ca. P. solani*' (Güldür *et al.*, 2018)
115 was used as positive control in the PCR studies with *tuf* and *vmp1* genes. Seven microlitres of
116 amplicons were electrophoresed in 1% of 1X TAE agarose gel, stained with ethidium bromide
117 and photographed under a UV transilluminator.

118 The nested-PCR products obtained using R16F2n/R16R2 and fTufAY/rTufAY primers
119 from symptomatic maize plants were analyzed by RFLP. Enzymatic digestions were carried
120 out in a final volume of 30 μ L including 2 μ L Fast Digest Green Buffer, 10 μ L amplicons and
121 1 U restriction enzyme *HpaII* (Thermo Scientific, Lithuania), according to the manufacturer's
122 instruction at 37°C for 20 min. The digested products were separated in 1.5% of 1X TAE
123 agarose gel, stained with ethidium bromide and photographed under a UV transilluminator.

124

125 **2.4 | Sequencing and phylogenetic relationships**

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

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

147

148 **3 | RESULTS**

149 The symptomatic maize plants, mainly located at the edge of the surveyed fields,
150 exhibited severe reddening symptoms in leaves and midribs, and showed atrophied
151 inflorescence and empty corn cob with poor quality of seeds (Figure 1). The use of P1/Tint
152 primer pair enabled to amplify DNA bands (about 1.6 kb) from the positive control and from
153 all symptomatic leaf tissues. However, no bands were observed from the asymptomatic plants
154 negative control (data not shown). The 16S rDNA partial sequences of samples Tr-AcM 1, -2,
155 -3 and -4 showed 100% identity with each other and 99.75% with that of '*Ca. P. solani*'
156 (GenBank accession number AF248959) and were submitted to GenBank under the accession
157 numbers MW344059, MW344060, MW344061, MW344062, respectively. The identification
158 was made with *iPhyClassifier* based on *in silico* RFLP patterns (similarity coefficient 1.00) and
159 this confirmed that the detected phytoplasma belong to the 16SrXII-A subgroup (Figure 2).
160 This identification was further supported by the clustering analysis with other '*Candidatus*
161 *Phytoplasma*' species and '*Ca. P. solani*' strains deposited to GenBank from Turkey and
162 neighboring countries (Figure 3).

163 For the *tuf* and *vmp1* genes, amplification of approximately 1.4 kb and 0.9 kb, was
164 obtained from the nested-PCR assays with TYPH10F1/R1 and fTufAY/rTufAY primer pairs,
165 respectively (data not shown). Restriction patterns of PCR products from the *tuf* gene with
166 *HpaII* were identical to each other and confirmed that all maize strains belong to *tuf*-type-b
167 (Figure 4). The primer pair TYPH10F1/R1 also amplified the expected length fragment in all
168 the maize samples and positive control but not from the negative controls. The *vmp1* amplicons
169 of Tr-AcM 1, -2, -3 and -4 samples were sequenced in both directions, and the sequences were
170 deposited in GenBank under the accession numbers MW984610, MW984611, MW984612,
171 MW984613, respectively. The unrooted phylogenetic tree (Figure 5) constructed using the
172 *vmp1* sequences listed in Table 1 and those obtained from corn from Turkey showed that the
173 nucleotide sequences of maize strains clustered according to the typology of the *RsaI*/RFLP

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

177

178 **4 | DISCUSSION**

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

187 The molecular characterization of ‘*Ca. P. solani*’ in maize by using the *tuf* and *vmpI* genes
188 confirmed the finding and the phytoplasma identity and represents a new epidemiological
189 information about ‘*Ca. P. solani*’ in Turkey. Based on the *tuf* gene characterization, all the
190 symptomatic maize samples showed the presence of only *tuf*-type b which agrees with reports
191 from Bosnia and Herzegovina (Kovačević *et al.* 2014; Delić *et al.*, 2016). *Tuf*-type b is
192 primarily associated with field bindweed (*Convolvulus arvensis* L.) (Langer and Maixner,
193 2004) that is its dominant reservoir plant species throughout most of the Europe and Turkey
194 (Ember *et al.*, 2011; Ozturk *et al.*, 2017). With the abundance of these plants, ‘*Ca. P. solani*’
195 has a strong potential to spread out to maize and other crop plants when the insect vector(s) are
196 present. Typing *vmpI* gene with *RsaI* digestion and sequence analysis indicated that all maize
197 strains in the present study belonged to the same molecular type named V15. It was previously
198 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.

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

235

236 **CONFLICT OF INTEREST**

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

238

239 **AUTHOR CONTRIBUTION**

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

245

246 **DATA AVAILABILITY STATEMENT**

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

249

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

386

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

394

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

402

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

406

407 **Figure 5.** Unrooted phylogenetic tree inferred from the ‘*Ca. P. solani*’ strain nucleotide sequences of
408 the *vmp1* gene. Minimum evolution analysis was carried out using the neighbor-joining method and
409 bootstrap replicated 1,000 times. The full names of the phytoplasma strains included in the phylogenetic
410 analysis are given in the Table 1. The GenBank accession number of each sequence is given in
411 parenthesis; gene sequences obtained in the present study are indicated with red squares. Square brackets
412 show the grouping obtained by digestion of the amplicons with the endonuclease *RsaI*.

413

414 **Figure 6.** Virtual RFLP patterns of *vmp1* gene sequence amplified with the primer pair TYPH10F/R of
415 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*' *vmp1* gene sequences available in GenBank used in the phylogenetic analysis.

421