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ORIGINAL ARTICLE

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Detection and seed transmission of Bermudagrass phytoplasma in maize in Turkey

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Abstract

During 2017, maize cultivation areas in the provinces of Adana and Kahramanmaraş (Turkey) were surveyed to inspect maize plants with symptoms similar to those associated with of phytoplasma disease, that is, yellowing, short internodes and small corncobs. Thirty fields were inspected and two hundred samples from symptomatic and asymptomatic plants were collected, together with insects considered as potential vectors of phytoplasmas. All samples were assayed by polymerase chain reaction (PCR) and subsequently analysed by restriction fragment length polymorphism and sequencing to identify the phytoplasmas detected in the plant material and insects. Results of laboratory assays and phylogenetic analyses showed that the Bermudagrass white leaf phytoplasma ('*Candidatus* Phytoplasma cynodontis') was present in both maize plants and seeds, showing 99% sequence identity with other reported phytoplasma strains from GenBank, whereas no PCR amplifications were obtained from tested insects. The seeds of infected plants, sown in an insect-proof screenhouse, produced plantlets that were found PCR-positive for the Bermudagrass white leaf phytoplasma, indicating its seed transmission.

KEYWORDS
16S rDNA, maize, PCR, phytoplasma, RFLP, transmission

1 | INTRODUCTION

Maize (*Zea mays* L.) is an annual plant in the *Poaceae* family and the most cultivated species in this family after wheat and rice worldwide. Maize can be grown in humid region, and 90% of the crop production is used for human and livestock feeding. Maize is grown in all the regions of Turkey, particularly in the two provinces of Adana and Kahramanmaraş that produce 1,274,428 tonnes and 615,247 tonnes, respectively, composing 7.2% of the Turkish production (Anonymous, 2016). During April and May 2016, several surveys were carried out in cornfields of Adana province, wherein maize plants showing leaf yellowing, short internodes, bushy growth, dwarfing and small corncob symptoms were observed. Bermudagrass [*Cynodon dactylon* (L.) Pers] and Johnson grass (*Sorgum halepense* (L.) plants, cohabiting with corn plants with white leaf symptoms, were also observed in the inspected fields. Molecular tests demonstrated that all these plants were infected by the Bermudagrass white leaf phytoplasma. This phytoplasma mainly affects monocotyledonous plants and corn in Turkey, and its presence is associated with streaks on the leaves ranging from light green to yellow, large chlorotic areas and white discolorations, axillary shoots proliferation, stunted growth, reduced leaf size, shortened stolon and rhizome death of symptomatic plants. These symptoms were yearly occurring in many different regions, threatening the maize plantation and production. Accordingly, this research was conducted to verify some of the epidemiological

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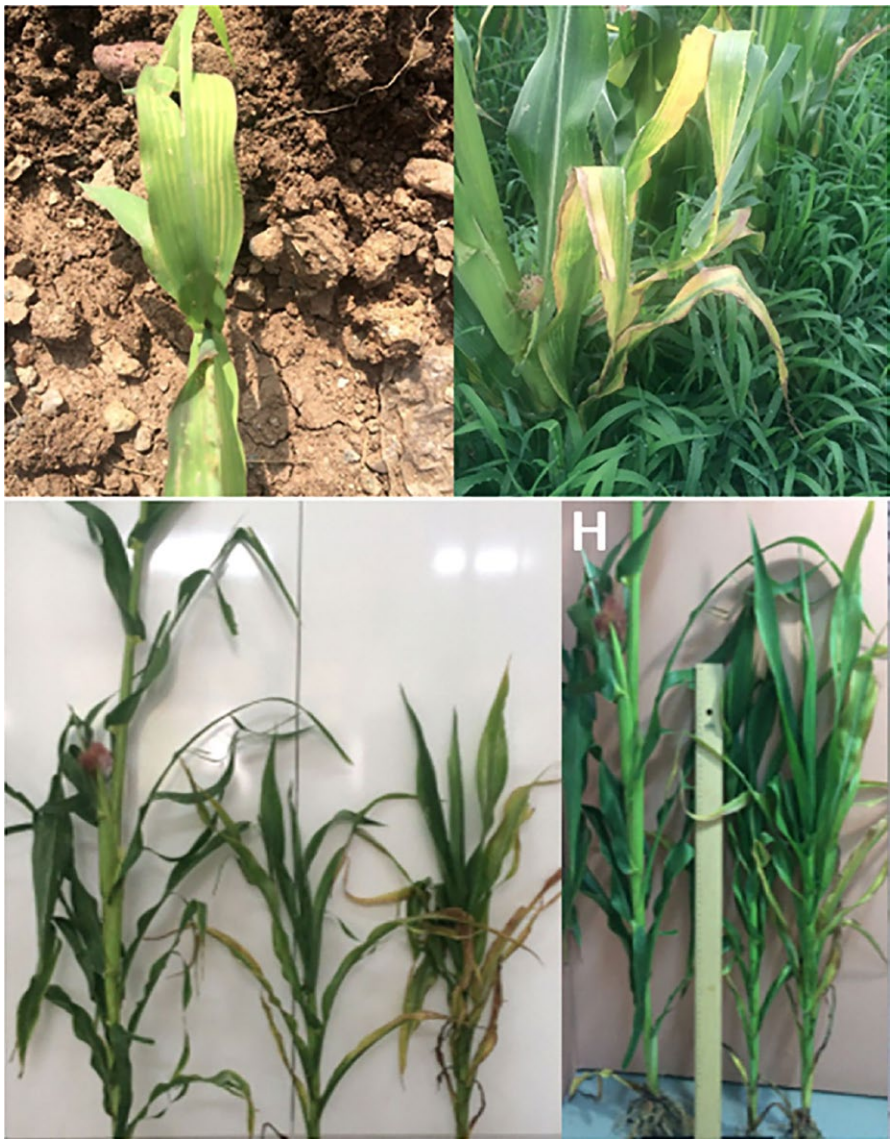


FIGURE 1 Maize plants in fields showing symptoms similar to those of phytoplasma, that is yellowing, bushy growth, short internodes and dwarfing. H: Healthy maize plant [Colour figure can be viewed at wileyonlinelibrary.com]

aspects of this disease in Turkey, that is, presence of potential insect vectors and seed transmission.

2 | MATERIALS AND METHODS

2.1 | Samples collection

During April and May 2017, repeated surveys with sample collection were carried out in maize fields with plants showing leaf discoloration, yellowing, short internodes and bushy growth (Figure 1). In total, two hundred samples were collected from 20 maize fields in Adana and 10 in Kahramanmaraş provinces (south of Turkey). Collected samples consisted of leaves, taken from symptomatic and asymptomatic plants. Furthermore, potential insect vectors of phytoplasma were collected from maize fields and nearing borders using a D-Vacuum apparatus. Afterwards, 2 months after the first survey, all fields were inspected again and corncobs were collected from the same plants that gave polymerase chain reaction (PCR)-positive results for the presence of phytoplasma.

2.2 | Extraction of DNA from maize and seeds

The DNA was extracted from different templates of diverse and origin, that is, seeds and midribs of fresh leaves from symptomatic and symptomless plants collected from different fields (Figure 1), together with insects, according to Ahrens and Seemüller (1992) procedure with some modifications. In brief, 1 g of tissue (midrib or seeds) was homogenized in 4 ml of CTAB buffer pH 8.0 (2% w/v cetyltrimethylammonium bromide, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, 0.2% 2-β-mercaptoethanol, 2% polyvinylpyrrolidone), followed by an incubation at 65°C for 30 min. After elimination of plant tissue residues by centrifugation at 13,000 rpm for 10 min, the nucleic acids in suspension were clarified through chloroform–isoamyl alcohol (24:1), the supernatant was recovered and left in suspension with isopropanol at −20°C overnight. The nucleic acids were precipitated 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 distilled water. The DNA was extracted from insects according to a protocol described by Doyle and Doyle (1990).

TABLE 1 List of collected and PCR-assayed samples for the presence of phytoplasma

Field No.	Village/Town/Province	Acronym	Symptoms (%)	Phytoplasma presence
1	Ayvalı/İmamoğlu/Adana	aia	1	Negative
2	Menteş/İmamoğlu/Adana	mia	1	Negative
3	Danacılı/İmamoğlu/Adana	dia	1–2	Negative
4	Koyunevi/İmamoğlu/Adana	kia	1	Negative
5	Saygeçit/İmamoğlu/Adana	sia	1	Negative
6	Körkuyu/Ceyhan/Adana	kca	2	Negative
7	Yeşilbahçe/Ceyhan/Adana	yca	2	Negative
8	Altıgöl/Ceyhan/Adana	aca	1	Negative
9	Mercin/Ceyhan/Adana	mca	2	Negative
10	İsalı Village/Ceyhan/Adana	ica	3	Negative
11	Gümüşyazı/Yüreğir/Adana	gya	3	Positive
12	Yerdelen/Yüreğir/Adana	yya	3	Positive
13	Şıhmurat/Yüreğir/Adana	sya	4	Positive
14	Danışment/Yüreğir/Adana	dya	3	Positive
15	Alihocalı/Yüreğir/Adana	aya	2	Positive
16	Camuzağılı/Seyhan/Adana	csa	3	Positive
17	Çaputçu/Seyhan/Adana	çsa	4	Negative
18	Köylüoğlu/Seyhan/Adana	ksa	3	Positive
19	Sarı Hamzalı/Seyhan/Adana	ssa	3	Negative
20	Büyük Dikili/Seyhan/Adana	bsa	1	Positive
21	Karaçay/Pazarcık/Kahramanmaraş	ptk	2	Positive
22	Osmandede/Pazarcık/Kahramanmaraş	opk	1	Negative
23	Ördekdede/Pazarcık/Kahramanmaraş	öpk	1	Negative
24	Karahüyük/Pazarcık/Kahramanmaraş	kapk	1	Negative
25	Emiroğlu/Pazarcık/Kahramanmaraş	epk	1	Negative
26	Tahtalıdedeler/Türkoğlu/Kahramanmaraş	ttk	1	Negative
27	Pınarhüyük/Türkoğlu/Kahramanmaraş	ptk	1	Negative
28	Özbek/Türkoğlu/Kahramanmaraş	ötk	1	Negative
29	Çobantepe/Türkoğlu/Kahramanmaraş	ctk	1	Negative
30	Göllühöyük/Türkoğlu/Kahramanmaraş	gtk	1	Negative

2.3 | Detection of phytoplasmas by polymerase chain reaction

The phytoplasma detection was carried out through the application of nested PCR assays, performed on DNA extracted from samples of different sources and templates, that is, midribs, corncob seeds and insects, and using primer pairs R16F1/R16R0 (Davis & Lee, 1993; Lee, Gundersen, Hammond, & Davis, 1994) and R16F2n/R16R2 (Gundersen & Lee, 1996), which amplify fragments of 1.4 and 1.25 Kbp from the 16S rRNA gene, respectively. Accordingly, 100 ng of DNA was used for amplification in a final volume of 50 µl of PCR mixture, containing 1 µl dNTPs (10 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 sterile distilled water. The PCRs were carried out for 35 cycles in a Techne TC 4,000 cycler (GMI, New York, USA) as follows: 1 min at 95°C, 2 min at 50°C and 3 min at 72°C.

The nested-PCR was applied on a 1:50 dilution of the mixture from the PCR, under the same conditions except for the annealing temperature that was 55°C. An extension step of 10 min at 72°C was used in both PCR assays. Ten microlitres of nested-PCR product was electrophoresed in 1.2% of 1X TAE agarose gel. DNA was stained with ethidium bromide and photographed under a UV transilluminator.

2.4 | Restriction fragment length polymorphism (RFLP)

Nested-PCR products obtained from infected maize plants and corncobs were analysed by RFLP. Enzymatic digestions were carried

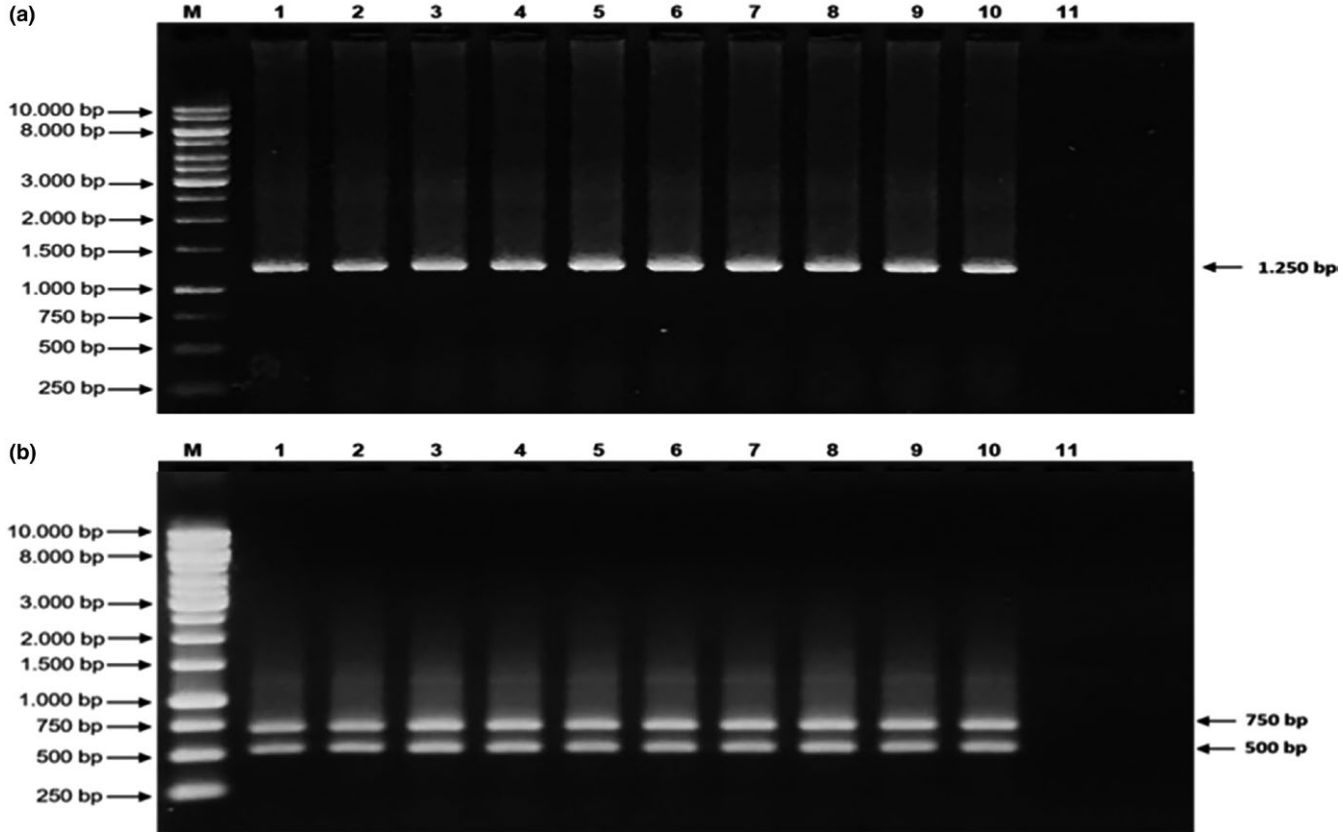


FIGURE 2 Agarose gel electrophoresis pictures showing (a) nested-PCR products and (b) RFLP profiles of nested-PCR amplicons digested with *Eco*RI, obtained from symptomatic maize plants. Samples: Lane 1: gya; lane 2: yya; lane 3: sya; lane 4: dya; lane 5: aya; lane 6: csa; lane 7: ksa; lane 8: bsa; lane 9: ptk; lane 10: M8 (Bermudagrass white leaf phytoplasma; GenBank accession number HE599395); lane 11: aia-healthy maize plant; lane M; marker 1 kb DNA ladder

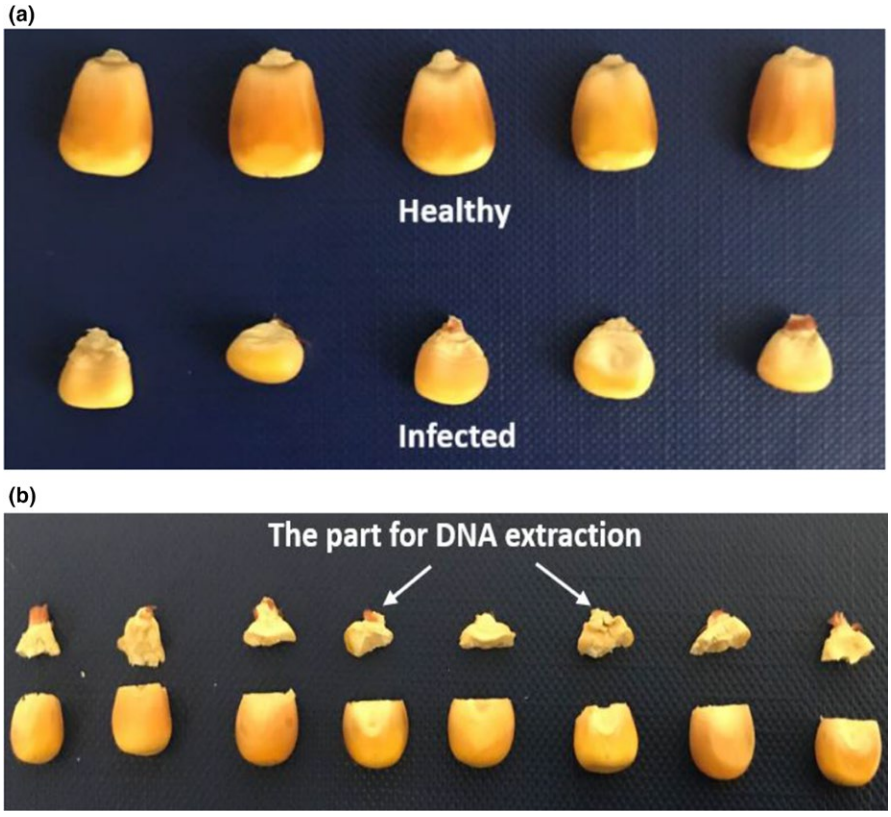


FIGURE 3 (a) Comparison between grains of corncobs taken from healthy and infected plants. (b) Tip cap of grain (embryo region) used for DNA extraction [Colour figure can be viewed at wileyonlinelibrary.com]



FIGURE 4 Seedlings germinated under greenhouse and showing deformed corncobs in which the '*Ca. P. cynodontis*' was detected in maize plants in inspected fields. (1) gya, (2) yya, (3) sya, (4) dya, (5) csa, (6) ksa, (7) ptk [Colour figure can be viewed at wileyonlinelibrary.com]

out in a final volume of 15 µl, using about 300 ng of amplicons and 0.25 µl of the restriction enzyme *Eco*RI (Thermo Scientific), to differentiate amplified DNA of phytoplasmas from that of host plant (Nejat, Sijam, Abdullah, Vadamalai, & Dickinson, 2009), following the manufacturer's instructions (Promega, Madison, USA). Digested amplicons were analysed by electrophoresis on a 1.5% 1X TAE agarose and visualized as described previously. Three additional endonucleases *Alu*I, *Hha*I and *Taq*I, were used for the identification of the ribosomal group of the phytoplasma detected in the infected samples, together with virtual RFLP analysis on the sequences by the *i*PhyClassifier (Zhao et al., 2009).

2.5 | Sequence and phylogenetic analyses

Nested-PCR amplicons, obtained from maize plants in the field and seedlings grown in the glasshouse, were excised from agarose gel, washed and eluted by centrifugation. Eluted DNA was sequenced in both directions (using primers R16F2n and R16R2) in an automated sequencer (ABI 3130xl Genetic Analyzer, Applied Bio. REFGEN Gen Araştırmaları ve Biyoteknoloji Ltd. şti. Ankara, Turkey). The complete sequence (1.25 Kbp) was obtained by assembling the sequences of both directions. The phylogenetic analysis was performed on nucleotides sequences of 43 '*Candidatus* Phytoplasma' species (Bertaccini & Lee, 2018) retrieved from the GenBank. MEGA6 software (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013) was used to construct sequence alignment, based on the Maximum Composite Likelihood method, whereas the phylogenetic tree was inferred from the Neighbor-Joining method (Saitou & Nei, 1987).

2.6 | Phytoplasma detection in seedlings derived from grains of infected plants

Two months after the first survey, seeds (20 per each plant) from mature corncob of eight maize plants found infected with phytoplasmas (seven from Adana and one from Kahramanmaraş) were collected (Table 1) and sown to germinate in a glasshouse under regular irrigation and fertilization. Moreover, insecticide was applied regularly to prevent possible insect-mediated transmission of phytoplasma inside the glasshouse. Once the development of corncobs of cultivated seedlings was completed, leaves and seeds were checked with PCR to verify whether phytoplasmas were still present.

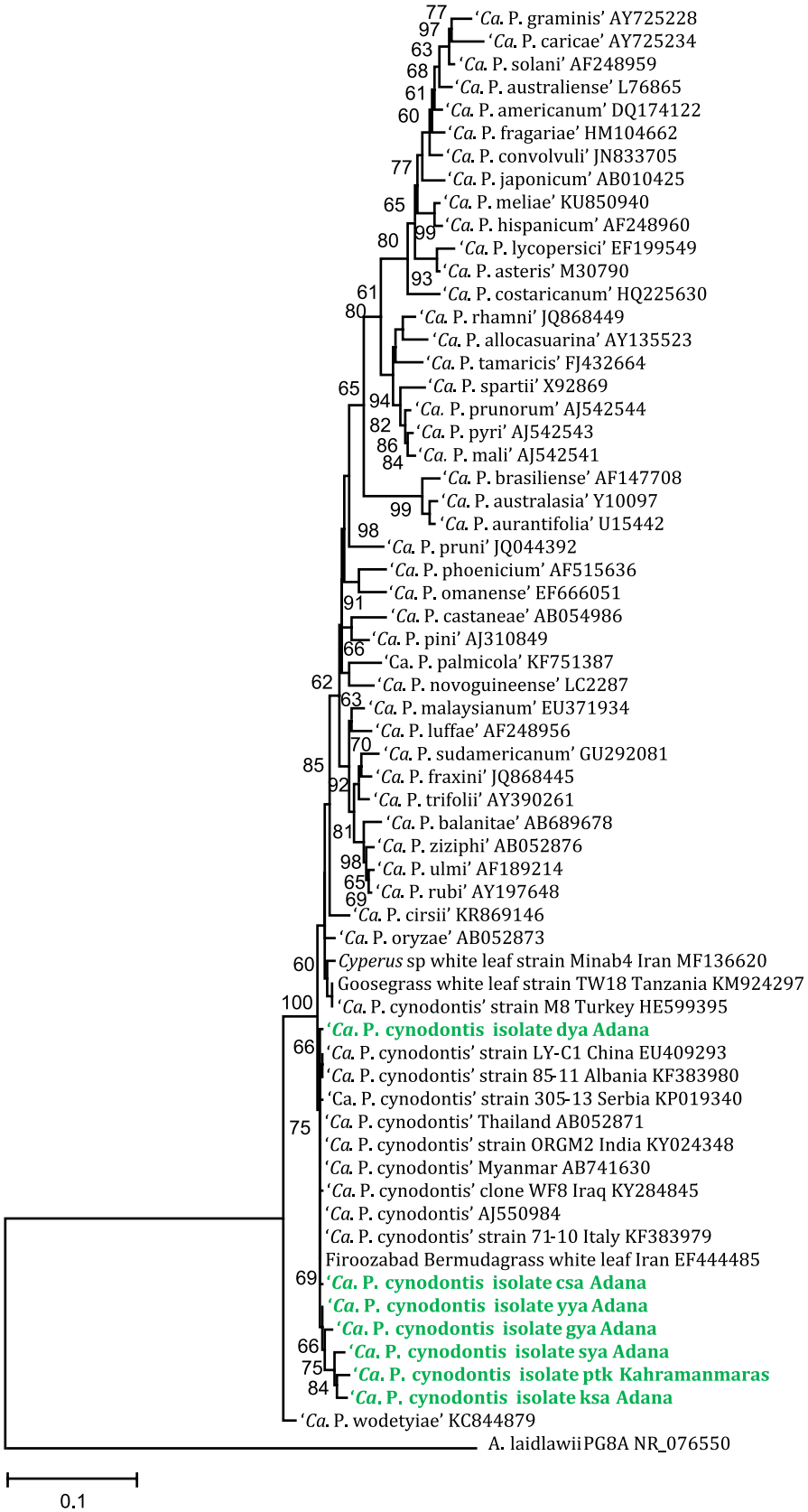


FIGURE 5 Phylogenetic tree constructed based on the Neighbor-joining method of the 16S rDNA sequences of phytoplasma strains detected in maize seedlings from infected mother plants (in green). *A. laidlawii* was used as outgroup to root the tree. Numbers at branch nodes indicate the percentage of support out of 1,000 bootstrap replications. Bootstrap values above 60% are shown. Scale bar represents 0.1 nt substitutions per site [Colour figure can be viewed at wileyonlinelibrary.com]

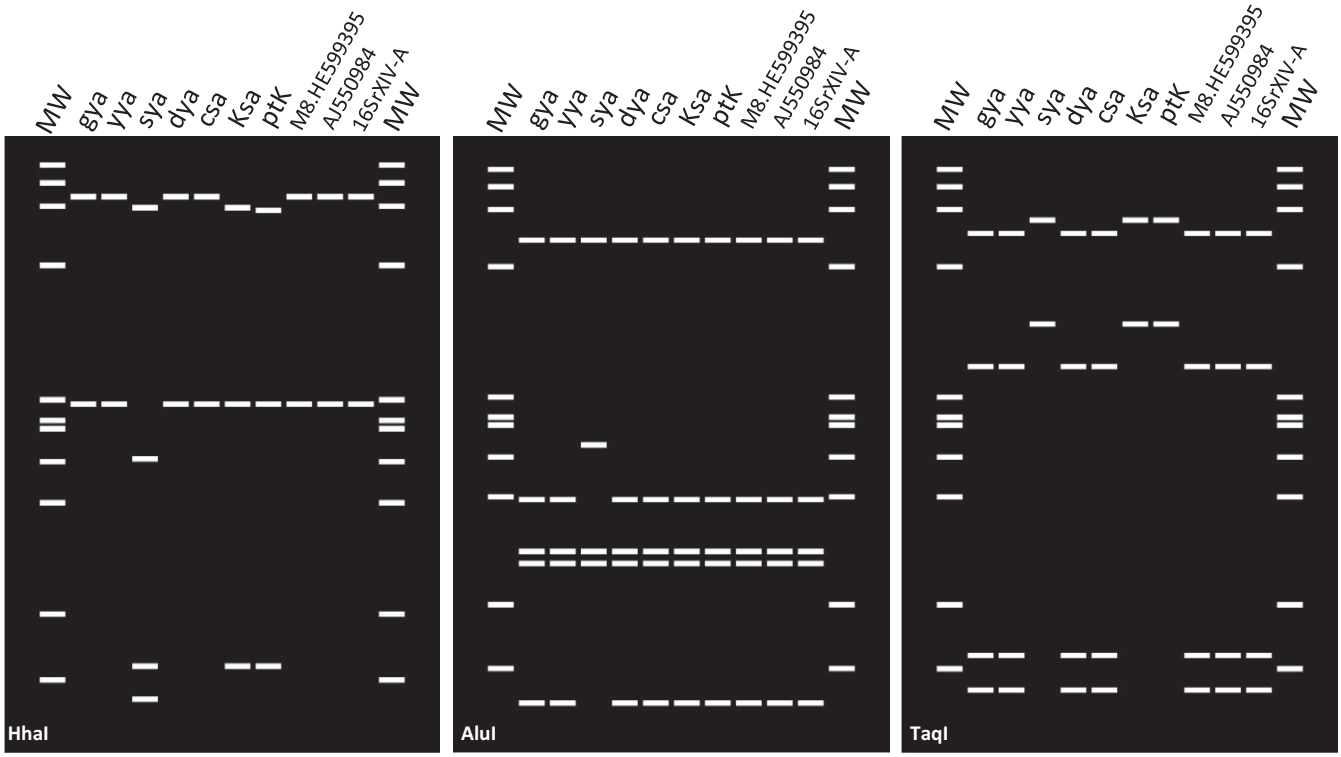


FIGURE 6 Profiles of virtual RFLP using *i*PhyClassifier on sequences obtained from phytoplasma-infected seedlings. Restriction enzymes used in RFLP are listed at the bottom of each picture. Sample acronyms are as reported in Table 1

3 | RESULTS AND DISCUSSION

Results of laboratory assays, conducted on samples collected from thirty maize fields (Table 1), showed the presence of phytoplasmas in nine sampled plants (eight from Adana, one from Kahramanmaraş province) that generated amplicons of the expected 1.25 kbp DNA band in nested-PCR (Figure 2a). These phytoplasma presence was confirmed by RFLP when their amplicons were digested with *Eco*RI that produced 500 and 750 bp DNA bands (Figure 2b). All symptomatic maize plants, showing leaf yellowing, short internodes, bushy growth, dwarfing, small corncobs, were found to host phytoplasmas, whereas no amplicons were obtained from asymptomatic corn samples, neither from *Empoasca decipiens* nor from *Cicadulina bipunctella* (Hemiptera; *Cicadellidae*) that were the only leafhopper species collected in the cornfields and in nearby ones.

Seeds collected from eight phytoplasma-positive plants (Figure 3) proved to be infected at the end of the growing season; seven out of the eight batches of plants in the glasshouse produced again infected cobs (Figure 4). Nested-PCR amplicons obtained from the positive samples were sequenced (GenBank accession numbers MH898651-MH898657). BLAST and phylogenetic analyses of sequences showed to be related to those of '*Candidatus* Phytoplasma cynodontis' (Figure 5). In addition, they proved to be closely related to the strain M8 previously reported from Turkey (GenBank accession number HE599395) (Çağlar, Satar, & Elbeaino, 2013a). The virtual RFLP analyses carried out using *i*Phyclassifier confirmed that the strains present in the plants derived from infected cobs in glasshouse were infected with a 16SrXIV group phytoplasma (Figure 6) (Lee, Gundersen-Rindal, Davis, & Bartoszyk, 1998). However, the virtual RFLP profiles of sequenced strains indicated that only three were different when compared to the phytoplasmas in 16SrXIV-A subgroup (Figure 6). In particular, the phytoplasma strain sya showed differences with *Hha*I, *Alu*I and *Taq*I restriction enzymes, while those of ksa and ptk showed polymorphisms with *Hha*I and *Taq*I (Figure 6). These polymorphisms could indicate a selection process carried out in the original phytoplasma population as already observed in other cases of phytoplasma seed transmission (Botti & Bertaccini, 2006). The phytoplasma seed transmission in maize was already reported in the literature for the "stolbur" phytoplasma ('*Ca.* P. solani') (Calari et al., 2011; Satta et al., 2017); however, the first seed transmission of '*Ca.* P. cynodontis' in maize was experimentally demonstrated in this study.

Bermudagrass white leaf (BGWL), a destructive disease of *C. dactylon* associated with the presence of phytoplasma, was first described in Taiwan (Chen, Lee, & Chen, 1972) and afterwards reported in a number of other countries in Asia (Khanna et al., 2015; Lee, Davis, & Gundersen-Rindal, 2000; Nejat et al., 2009; Rao et al., 2007; Zahoor, Bashir, Nakashima, Mitsueda, & Murata, 1995), Africa (Dafalla & Cousin, 1988), Cuba (Arocha et al., 2005), Australia (Padovan et al., 1999; Tran-Nguyen, Blanche, Egan, & Gibb, 2000), Europe (Marcone, Schneider, & Seemüller, 2004; Mitrović et al., 2015), Iran (Salehi, Izadpanah, Siampour, & Taghizadeh, 2009) and recently in Turkey (Çağlar et al., 2013a). Bermudagrass white leaf phytoplasma was experimentally root-bridge transmitted to wheat plants in Turkey (Çağlar, Satar, & Elbeaino, 2013b) where it seems to seriously threaten the cultivation of maize, although potential insect vectors were not identified in this research.

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