



Detection and molecular characterization of two genetically distinct phytoplasmas associated with mungbean seed pod abortion in Iran

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Abstract

New symptoms including aborted seed pods disease were observed on mungbean (*Vigna radiata*) plants in the most important growing areas in Iran that was similar to symptoms of phytoplasma disease previously reported from Australia. Thirty samples were collected from symptomatic and non-symptomatic plants, followed by PCR amplification, DNA sequencing, RFLP and phylogenetic analysis of 16S ribosomal RNA gene to detect and identify possible associated phytoplasmas. Results indicated that two different phytoplasmas, related to ‘*Candidatus Phytoplasma trifolii*’ and ‘*Candidatus Phytoplasma aurantifolia*’, were associated with different symptomatic plants samples from the same field, mixed infection was not observed in these samples. The sequence analysis results indicated that the nucleotide sequences from mungbean isolates 47 (MT674290), 7 (MT674291) and 32 (MT674292) had 98.8% sequence identity to ‘*Candidatus Phytoplasma aurantifolia*’ isolate TBBP-H1 (Acc. No: MN565885), while isolates 80 (MT674293) and 25 (MT674294) had 99.8% sequence identity similar to ‘*Candidatus Phytoplasma trifolii*’ (MF092789). In silico RFLP and phylogenetic analyse of mungbean seed pod abortion (MubSpa) phytoplasma indicated that the associated phytoplasmas belonged to phytoplasmas of subgroups 16SrVI-A and 16SrII-D. To the best of our knowledge, this is the first report of association ‘*Ca. P. trifolii*’ and ‘*Ca. P. aurantifolia*’ strains infecting mungbean plants in Iran.

Keywords Mungbean · Seed pod abortion · *Candidatus Phytoplasma trifolii* · *Candidatus Phytoplasma aurantifolia*

Introduction

Mungbean (*Vigna radiata*), known as Mash in Iran, is mainly cultivated in East Asia, Southeast Asia and the Indian subcontinent (Smartt 1990). The mungbean has been domesticated in Persia (Iran), where its progenitor, *Vigna radiata* subspecies *sublobata*, can be found (Tomooka et al. 2003; Fuller 2007). Mungbean is infected with different plant pathogens, such as phytoplasmas that reduce yield by

causing phyllody (Akhtar et al. 2010). A 16SrII-D phytoplasma, related to ‘*Candidatus Phytoplasma aurantifolia*’, was found to be associated with mungbean phyllody (Andhra Pradesh, India), which had typical symptoms of a phytoplasma disease including dwarf leaf, proliferation, stunting and phyllody (Ragimekula et al. 2014). Phytoplasmas are plant phloem restricted pathogens that are transmitted by insect vectors (Hajong et al. 2017). Phytoplasmas cause diseases in crops such as mungbean in Iran. Recently, new symptoms have appeared in mungbean fields in the vicinity of a soybean field that had symptoms similar to Soybean Seed Pod Abortion disease (SbpSpa) (Ghayeb Zamharir and Aldaghi 2018) called Mungbean Seed Pod Abortion (MubSpa). In this study, aborted seed pods were observed in the infected plants, which remained green while nearby unaffected crops matured as normally expected. The severely affected crops had few filled pods that they were uneconomical to harvest. The symptoms observed in MubSpa in Iran was similar to those reported in mungbean in Australia that was associated with phytoplasma and Soybean Seed Pod Abortion disease (SbpSpa) (Ghayeb Zamharir and Aldaghi

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2018). The goal of this research was to identify the possible phytoplasma strains associated with MubSpa symptoms observed in mungbean fields in Iran.

Materials and methods

Plant material and DNA extraction

Twenty-five leaf samples from MubSpa symptomatic plants were collected from mungbean fields located in Golestan province of Iran (36.7941° N, 54.1103° E) in 2019. Five leaves from non-symptomatic plants from the same fields were used as the negative control. The total genomic DNA was extracted from 0.5 g of leaf midrib tissue based on Doyle and Doyle (1987) method. The DNA samples were analyzed in 1% agarose gels, quantified using a NanoDrop spectrophotometer and adjusted to a suitable concentration (100 ng per µl) for further tests.

Detection of phytoplasma

Two phytoplasma primer sets, P1/Tint (Deng and Hiruki 1991; Smart et al. 1996) and R16F2n/R16R2 (Gundersen and Lee 1996), were used as direct and nested primers, respectively, to detect phytoplasmas by the amplification of a 1250 bp 16S rRNA-encoding gene F2R2 fragment, to detect phytoplasmas by the amplification of a 16S rRNA-encoding gene F2R2 fragment of 1250 bp. Reactions were performed in a 25 µl reaction mixture consisting of 1 unit of Taq polymerase (SinaGene, Tehran, Iran), 2.5 µl of 10XPCR Buffer (SinaGene, Tehran, Iran), 0.5 µM of each primer, 0.4 mM dNTPs, 3 mM MgCl₂ and 100 ng of total DNA. For PCR amplification, 35 cycles were carried out in an automated thermal cycler (BioRad, MyCycler™ Thermal Cycler System) with the following program: for direct PCR, the DNA was amplified by 35 cycles consisting of denaturation at 94 °C for 60 s (5 min for cycle 1), annealing at 53 °C for 2 min, and primer extension at 72 °C for 3 min (5 min for cycle 35). For nested amplification, the thermal conditions were the same except the annealing was at 55 °C for 2 min. The PCR products resulting from P1/Tint amplifications were diluted 1:30 with sterile deionized water and 1 µl of each dilution was then used as a template in the nested PCR using primers R16F2n/R2. The PCR products were electrophoresed in 1.2% agarose gels in a TAE buffer and visualized with a UV transilluminator following ethidium bromide staining.

DNA sequencing and phylogenetic analysis

Selected R16F2n/R2 (1,248 bp) amplified fragments from phytoplasma that were detected in mungbean plants (7, 25,

32, 47,80) were directly Sanger sequenced. The obtained 5 sequences were assembled, aligned and compared with nucleotide sequences in the GenBank database, using BLAST (BLASTN Ver. 2.2.18) (National Center for Biotechnology Information, Bethesda, MD, USA). Sequence alignments were done using ClustalX (Thompson et al. 1997). Phylogenetic analyses were inferred by using the Tamura-Nei model (Tamura and Nei 1993) with Maximum Parsimony analysis/method using 16S rRNA-encoding gene sequence from positive samples (7, 25, 32, 47, 80) and from 23 ‘*Candidatus phytoplasma*’ strains. The tree with the highest log likelihood (-1092.11) is used. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016). The analysis was replicated 1000 times. A bootstrap analysis was performed to estimate the stability and support for the inferred clades (Tamura et al. 2004).

RFLP analysis

Virtual restriction fragment length polymorphism (RFLP) analysis was performed on all of the 16S rRNA gene F2nR2 fragment generated in this study using the *iPhyClassifier* tool (Zhao et al. 2009). Each amplified 16S rRNA-encoding gene F2nR2 fragment was digested in silico with 17 restriction enzymes (*AluI*, *BamHI*, *BfaI*, *BstUI*, *DraI*, *EcoRI*, *HaeIII*, *HhaI*, *HinfI*, *HpaI*, *HpaII*, *KpnI*, *Sau3AI*, *MseI*, *RsaI*, *TspI* and *TaqI*) that were used for the phytoplasma 16S rRNA-encoding gene F2R2 fragment RFLP analysis (Wei et al. 2007). The virtual RFLP patterns, produced by strains obtained from mungbean, were compared with each other and with representatives of subgroups, within 16SrII and VI groups, using a Perl program developed by Wei et al. (2008).

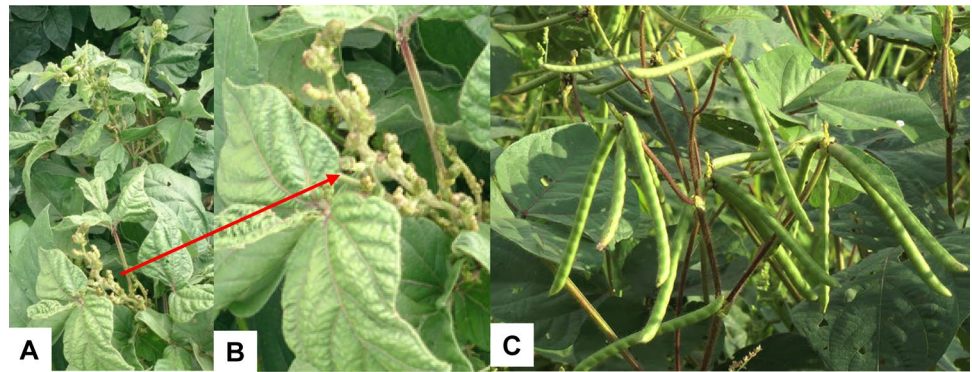
Results

Identification of phytoplasmas associated with MubSpa in mungbean

The aborted seed pod symptoms were observed in the mungbean fields in Golestan provinces (36.7941° N, 54.1103° E) in Iran. The symptomatic plants remained green in mature phase that they were uneconomical to harvest (Fig. 1). These symptoms were observed in 50% plants in every field.

The expected fragment size of 1.25 kb was amplified from a total of 24 out of 25 mungbean symptomatic samples. All of asymptomatic samples were negative in nested PCR analysis. The selected positive samples were sequenced and submitted to GenBank (Acc. No.: MT674290 to MT674294). The sequence analysis results indicated

Fig. 1 Symptoms of mungbean seed pod abortion disease (a), Close up of seed pods (b) pointed by red arrow compared with healthy one (c)



that the nucleotide sequences from mungbean isolates 47 (MT674290), 7 (MT674291) and 32 (MT674292) had 98.8% sequence identity with '*Candidatus Phytoplasma aurantifolia*' isolate TBBP-H1 (Acc. No: MN565885), while isolates 80 (MT674293) and 25 (MT674294) had 99.8% sequence identity with '*Candidatus Phytoplasma trifolii*' (MF092789).

Phylogenetic analysis

The aligned sequences of the mungbean phytoplasma isolates cluster with 22 phytoplasma sequences were obtained from GenBank. The mungbean isolates 47, 7 and 32 were classified with '*Ca. P. aurantifolia*' into 16SrII group (Fig. 2). In the VI branch of 16Sr group, the mungbean isolates 25 and 80 were clustered along with the previously characterized '*Ca. P. trifolii*'. The percentage of trees in which the associated taxa clustered together was shown next to the branches. The analysis involved 22 nucleotide sequences. The results indicated that two distinct phytoplasma isolates, related to 16SrII and 16SrVI groups, were associated with MubSpa disease in Iran.

RFLP analysis

A virtual RFLP pattern, derived from the queried 16S rRNA-encoding gene F2nR2 regions of mungbean isolates 25 and 80, indicated that these sequences were classified into 16SrVI-A sub-group (Fig. 3) (GenBank accession EU346380, 16SrVI-A), with a similarity coefficient ranging from 0.99 to 1.00. The phytoplasmas were members of 16SrVI-A (GenBank accession: EU346380, 16SrVI-A). The queried of 16S rRNA-encoding gene sequences of isolates 7, 32 and 47 all shared 99.1% similarity with '*Candidatus Phytoplasma aurantifolia*' reference strain (GenBank accession: U15442, 16SrII-B). The phytoplasma isolates are then identified as a '*Ca. P. aurantifolia*'-related strain. '*Ca. P. aurantifolia*'-related strain with F value 1 for virtual RFLP analysis (Fig. 3).

Discussion

Phytoplasmas are phloem-limited pleomorphic prokaryotes causing diseases in many plant species. Symptoms in legume plants include dwarf leaves and phyllody. In most of the cases, phytoplasmas infect legumes in the late stage of plant growth (Bertaccini and Duduk 2009; Bertaccini et al. 2014; Rao et al. 2011, 2017). Recently, there have been many reports that confirmed the association of phytoplasmas with emerging diseases in different host plants worldwide including Soybean Seed Pod Abortion disease that was caused by a phytoplasma belonging to 16SrVI groups (Ghayeb Zamharir and Aldaghi 2018) and mungbean phyllody (Andhra Pradesh, India) that caused by a phytoplasma related to '*Candidatus Phytoplasma aurantifolia*' (16SrII-D (Ragimekula et al. 2014).

This study revealed that two distinct phytoplasma isolates, that belonged to '*Ca. P. aurantifolia*' and '*Ca. P. trifolii*', were associated with MubSpa disease in Iran. Different RFLP patterns, similar to the strains classified in the '*Ca. P. aurantifolia*' and '*Ca. P. trifolii*' (Fig. 2), were obtained based on the current taxonomic scheme (Lee et al. 2004; Wei et al. 2008; Zhao and Davis 2016) and computer-simulated restriction analyses carried out on R16F2n/R16R2 sequences from five MubSpa phytoplasma strains in Golestan province (Iran). The results obtained by RFLP/gel electrophoresis analysis (Fig. 3) showed that MubSpa phytoplasma strains belonged to 16SrII and 16SrVI groups. Furthermore, the phylogenetic analyses of 16S rRNA-encoding gene F2R2 fragment in this study indicated the divergence between the MubSpa phytoplasma strains in Iran. These results showed similarity in ecological niches of various phytoplasma isolates (Johannesen et al. 2008; McCoy et al. 1989).

Mixed infections may occur in insect vectors or in plant host of phytoplasmas where are grown on dense and mixed farms. Since the host plant and the vector insect are not equally susceptible to phytoplasma infection, mixed infection of phytoplasmas could be different in the host plants or insect vectors (Lee et al. 1998; Gundersen et al. 1996). Interestingly, the infected mungbean fields, studied in this

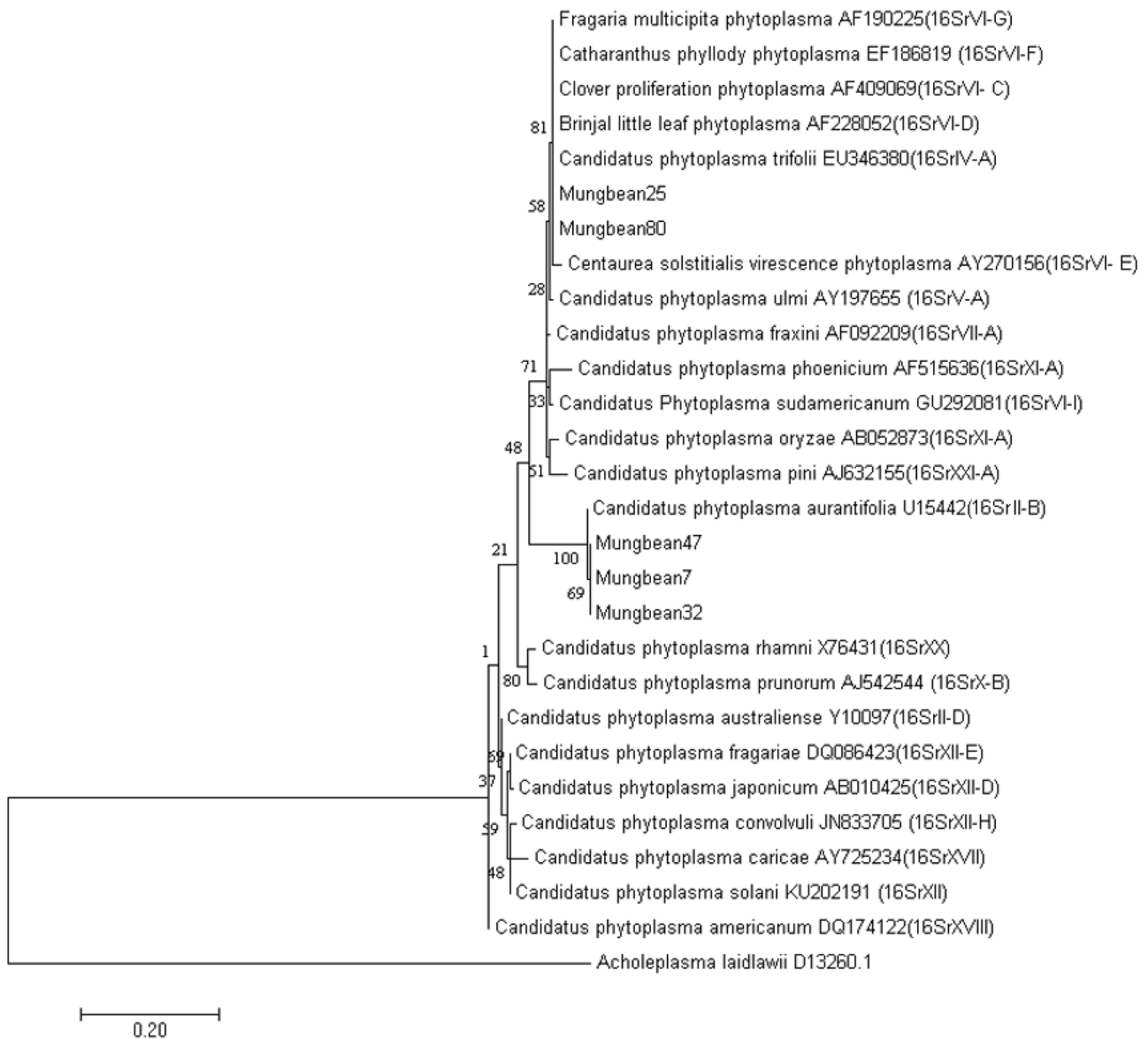


Fig. 2 Evolutionary analyses conducted in MEGA7 (Tamura et al. 2013) using the Maximum Likelihood method. The percentage of replicate trees in which the associated taxa clustered together in the

bootstrap test (1000 replicates) are shown next to the branches. Only values above 50 were reported

research, were near soybean fields infected with SbpSpa phytoplasma that belonged to 16SrVI groups (Ghayeb Zamharir and Aldaghi 2018). The results of this study showed that SbpSpa and MubSpa diseases were caused by *Ca. P. trifolii*-related strains that shares 99.8–99.9% sequence similarity with *Ca. P. trifolii*' reference strain (MF092789).

The feeding habits of insect vectors and plant host susceptibility to the phytoplasma could affect mixed infection of phytoplasmas in plant hosts. For example, many insect vectors of phytoplasma strains in the groups I or X of phytoplasmas are polyphagous. Since, different plant hosts could be feeding

by these common vectors, multiple infections of phytoplasma could occur and create a new ecological niche for the phytoplasma (Lee et al. 1998; Gundersen et al. 1996). Many studies indicated that the variety and number of weeds and plants, hosting the phytoplasmas inside and around fields, could strongly affect the life cycle and behavior of insect vectors, conceivably altering their host plant feeding preferences and the selection of phytoplasma strains (Johannesen et al. 2008). It is possible that same vector(s) transmit(s) *Ca. P. trifolii*-related strains from soybean or weeds to mungbean or vice versa. More studies are need to confirm this hypothesis.

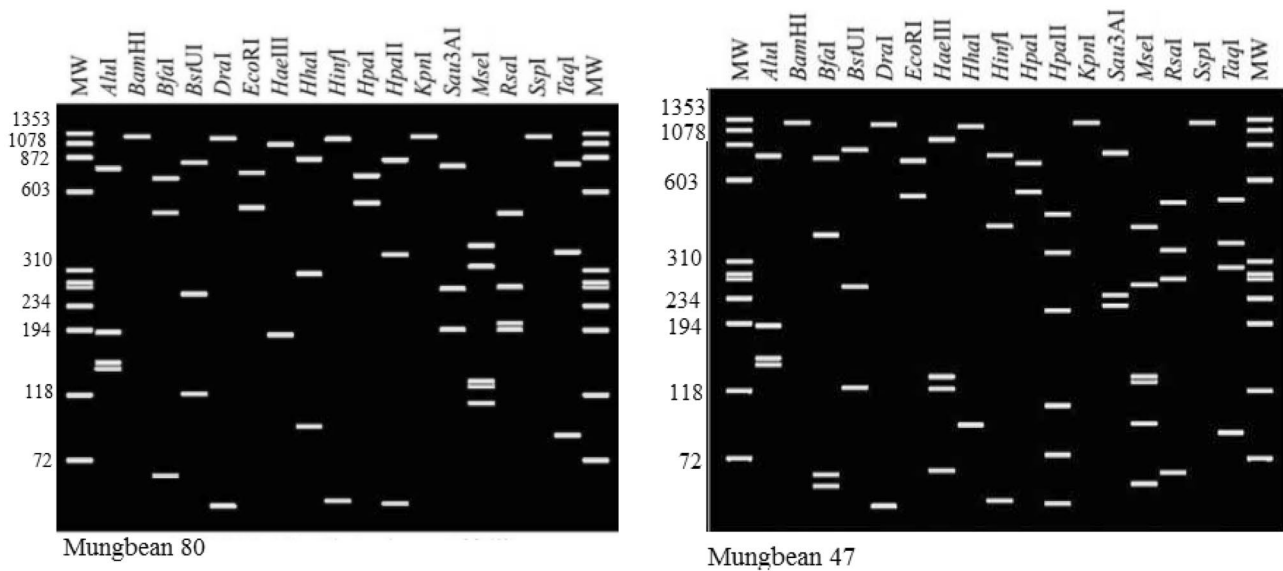


Fig. 3 The virtual pattern was generated by analysis of the R16F2n/R16R2 amplicon DNA sequences 47 and 80 using iPhyClassifier

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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