Phytopathologia Mediterranea
The international journal of the
Mediterranean Phytopathological Union

Firenze University Press
www.fupress.com/pm

Short Notes

Molecular detection and identification of a '*Candidatus* Phytoplasma solani'-related strain associated with pumpkin witches' broom in Xinjiang, China

Xu WANG[1], Chun-Guang WANG[1,*], Xiao-Yan LI[2], Zheng-Nan LI[2,*]

[1] *College of Mechanical and Electrical Engineering, Inner Mongolia Agricultural University, Hohhot 010018, China*
[2] *College of Horticulture and Plant Protection, Inner Mongolia Agricultural University, Hohhot 010018, China*
*Corresponding authors. E-mail: jdwcg@imau.edu.cn; lizhengnan@imau.edu.cn

Summary. Pumpkin plants showing symptoms of witches' broom (PuWB) were observed in Xinjiang Uyghur autonomous region, China, in September 2018. A phytoplasma was detected in symptomatic plants by PCR amplifying portions of the 16S ribosomal and *tuf* genes. In addition, the phylogeny based on these genes sequencing indicated that the PuWB strain clusters with '*Candidatus* Phytoplasma solani' (subgroup 16SrXII-A). Furthermore, based on *in silico* and *in vitro* restriction fragment length polymorphism analyses, the PuWB phytoplasma was confirmed as a '*Ca.* P. solani'-related strain. This was the first record of the occurrence of phytoplasma presence in pumpkins in China, and the first record of 16SrXII phytoplasma infecting pumpkins in the world.

Keywords. *Cucurbita moschata*, pathogen, phylogeny, RFLP.

Citation: X. Wang, C.-G. Wang, X.-Y. Li, Z.-N. Li (2021) Molecular detection and identification of a '*Candidatus* Phytoplasma solani'-related strain associated with pumpkin witches' broom in Xinjiang, China. *Phytopathologia Mediterranea* 60(1): 63-68. doi: 10.36253/phyto-11066

Accepted: October 21, 2020

Published: May 15, 2021

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

Editor: Assunta Bertaccini, Alma Mater Studiorum, University of Bologna, Italy.

INTRODUCTION

Phytoplasmas are prokaryotic plant pathogens first discovered in 1967 (Doi *et al.*, 1967). They are wall-less *Mollicutes* phylogenetically related to low G+C Gram-positive bacteria (Weisburg *et al.*, 1989). Phytoplasma presence is associated with host symptoms such as phyllody, virescence, and witches' broom (Hogenhout and Segura, 2010). Due to difficulties in establishing axenic cultures of these organisms (Contaldo *et al.*, 2012; Contaldo *et al.*, 2016; Contaldo and Bertaccini, 2019), the methods routinely used for bacterial diagnosis and taxonomy are not yet applicable to phytoplasmas. These organisms are classified in '*Candidatus* Phytoplasma', according to IRPCM (2004). Based on similarities in restriction fragment length polymorphism (RFLP) patterns of 16S rRNA genes, phytoplasmas are also classified into 16Sr groups and subgroups. For the recognition of a new subgroup, the similarity coefficient among the RFLP patterns should be 0.97 or lower (Wei

et al., 2008); for a new group, the threshold value is 0.85 (Zhao *et al.*, 2009). In addition, for finer differentiation of closely related phytoplasma strains, some other genetic *loci* are used, such as the ribosomal protein (*rp*) genes and the elongation factor Tu (*tuf*) genes (Schneider *et al.*, 1997; Lee *et al.*, 1998). More than 30 16Sr groups (16SrI to 16SrXXXVI) and 43 '*Ca.* Phytoplasma' species have been established to date (Bertaccini and Lee, 2018; Bertaccini, 2019). The 16SrXII group, also known as "stolbur", includes '*Ca.* P. australiense', '*Ca.* P. convolvuli', '*Ca.* P. fragariae', '*Ca.* P. japonicum', and '*Ca.* P. solani' (Zhao and Davis, 2016).

Pumpkins (*Cucurbita moschata*) are grown for various uses. Their fruit has high vitamin, amino acids, polysaccharide, fibre and mineral contents, and are extensively used as vegetables, processed food and stock feed in different parts of the world (Kumar *et al.*, 2018). Pumpkin seeds are low in fat and rich in proteins, providing highly nutritional and health protective value for humans (Quanhong and Caili, 2005; Devi *et al.*, 2018). As one of the leading producers of *Cucurbita* spp., China had total production of 7,789,437 tonnes of pumpkins, squash and gourds in 2016 (http://www.fao.org/faostat/en/#data/QC/visualize).

The present study reports pumpkin disease with witches' broom as the main symptom, which occurred in Xinjiang Uyghur autonomous region of China. In this region, viruses, including *Cucumber mosaic virus* and *Zucchini aphid-borne yellows virus*, have been reported as the main pathogens on *Cucurbita* species (Cheng *et al.*, 2013; Peng *et al.*, 2019).

MATERIALS AND METHODS

Samples collection

In September 2018, during a survey on pumpkin diseases in Urumqi, Xinjiang, China, pumpkins (*C. moschata*) with symptoms indicative of phytoplasma presence were observed at a vegetable plot (ca. 1,300 m^2), with incidence of approx. 1.5%. The infected plants had small leaves, short internodes, growth of multiple leaves at each node, and delayed or no fruit development (Figure 1). The disease was named pumpkin witches' broom (PuWB). Samples were collected from symptomatic and asymptomatic plants for phytoplasma detection.

Amplification and sequencing phytoplasma gene specific regions

To detect phytoplasma presence in affected plants, the region encompassing the 16S rRNA, 16S-23S internal transcribed spacer region and partial 5' end of the 23S rRNA genes, and the partial *tuf* gene of phytoplasmas were amplified using PCR. Fresh leaves of the PuWB samples were collected and total nucleic acid extraction was performed using a cetyltrimethylammonium bromide-based (CTAB) method (Kollar *et al.*, 1990). Leaves of asymptomatic pumpkins were used as a negative control. The extracted nucleic acids were then used as templates in PCR assays. The PCR reactions were carried out using rTaq (TaKaRa). The reaction mixture consisted of 2.5 µL of 10× PCR buffer, 1 µL of each primer (10 µM), 2 µL of dNTPs (2.5 mM each), 0.5 µL of the DNA polymerase (5 U μL^{-1}), and 1 µL of DNA template (20 ng μL^{-1}), with added sterilized distilled water up to a final volume of 25 µL. The primers P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) followed by the primers R16F2n/R2 (Gundersen and Lee, 1996), amplifying the partial 16S rRNA gene for RFLP analyses, were used in nested PCR; 1 µL of 1:30 sterilized distilled water diluted PCR products from the first amplification was used as template in the nested PCR reactions. The PCR amplification cycling was set up for P1/P7, as described by Lee *et al.* (1998), and for R16F2n/R2, as described by Nejat *et al.* (2010). The primers fTufu/rTufu (Schneider *et al.*, 1997) were used to amplify the partial *tuf* genes, using 94°C for 3 min, 35 cycles each of 94°C for 30 s, 50°C for 30 s and 72°C for 1 min, and a final extension at 72°C for 10 min.

The PCR products of the expected sizes were purified using a QIAquick gel extraction kit (Qiagen), ligated onto pMD18-T vector (TaKaRa), and competent cells of *Escherichia coli* strain JM109 were transformed. Colonies containing recombinant plasmids were screened

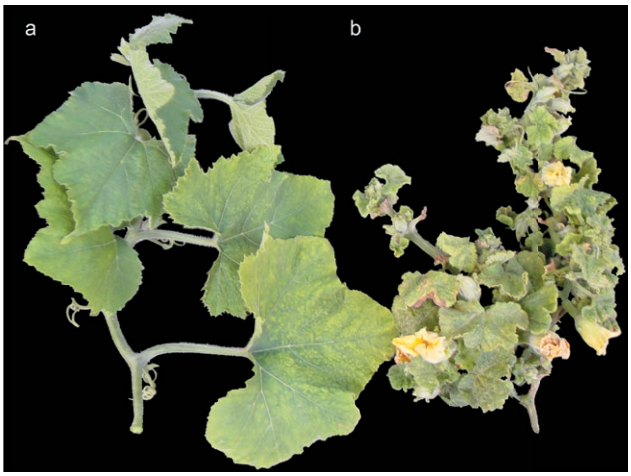

Figure 1. Symptoms on pumpkin. a) a healthy pumpkin vine; b) pumpkin vine exhibiting witches' broom symptom.

using agar plates of LB medium containing ampicillin (100 μg mL^{-1}) and X-Gal. The inserts were sequenced by TaKaRa. The M13 forward primer (-47): CGCCAGGGTTTTCCCAGTCACGAC and M13 reverse primer (-48): AGCGGATAACAATTTCACACAGGA were used for sequencing the inserts from both ends, which gave approx. 1.5 times of coverage for P1/P7 amplicons and 2 times of coverage for the *tuf* gene. For each gene from each sample, the inserts of three colonies were selected for sequencing.

RFLP and phylogenetic analyses

To identify the detected phytoplasma, RFLP patterns of the R16F2n/R2 region were analyzed, and phylogeny was determined based on sequences of partial ribosomal and *tuf* genes. To re-construct the phylogenetic tree, the 16S partial ribosomal sequences of phytoplasmas enclosed in subgroups 16SrXII-A to -K, and in another 11 ribosomal groups, were selected. A total of 15 *tuf* gene sequences from phytoplasmas in group 16SrXII and two from group 16SrI were used to infer the tree of *tuf* genes. Phylogenetic trees were built using the software MEGA7.0, by aligning sequences with ClustalW and using the neighbour-joining method with a bootstrapping of 1000 pseudoreplicates. In virtual RFLP analysis, the sequence of the R16F2n/R2 region of the detected phytoplasma was submitted to the online tool *i*Phyclassifier (https://plantpathology.ba.ars.usda.gov/cgi-bin/resource/iphyclassifier_legacy.cgi) for virtual gel image production and similarity coefficient calculations. The gel purified PCR products of the R16F2n/R2 amplicons were digested with the *Alu*I restriction enzyme according to the manufacturer's instruction (TaKaRa) and separated in a 2% agarose gel.

RESULTS AND DISCUSSION

Using the primers P1/P7, PCR fragments of approx. 1.8 kb were amplified from two PuWB samples (PuWB1 and PuWM2), which were of identical size to the P1/P7 region of phytoplasmas. The subsequent nested PCR used the primers R16F2n/R2 generated fragments of approx. 1.2 kb. PCR products of approx. 0.8 kb were of identical size to the partial *tuf* gene of phytoplasmas and were produced with the primers fTufu/rTufu. After cloning and sequencing, six sequences of the P1/P7 region (three from the PuWB1 and three from the PuWB2) were identical to each other, and were each of size 1,783 bp. The consensus sequence was deposited in GenBank under the accession number MH731275.

Six single colonies (three for PuWB1 and three for PuWB2) of the 0.8 kb fragment were also sequenced. The three sequences from the PuWB1 were identical to each other, and the consensus sequence was deposited under the accession number MK770825. One of the three sequences from the PuWB2 showed two site polymorphisms at nucleotide positions 62 and 758 with the other two, and this was deposited under the accession number MK770826. The consensus sequence for the other two strains was deposited under the accession number MK770827. All the three *tuf* gene sequences were of size 842 bp and had 99.8% similarity (840 bp / 842 bp) to each other. BLASTn searches for homologous gene sequences indicated that the obtained P1/P7 region sequence was homologous to those of phytoplasmas classified in the 16SrXII group, having the greatest nucleotide sequence similarity of 99.5% (1772 nt / 1781 nt) with '*Ca*. P. solani' strain SX-CP from *Salvia miltiorrhiza* from Shanxi Province, China (GenBank accession number KT844648). The *tuf* gene sequences showed the greatest nucleotide sequence similarity 99.6% (840 nt / 843 nt) to that of '*Ca*. P. solani' strain PFY from paper flower from China (GenBank accession number KC481242).

On the phylogenetic tree of the 16S ribosomal gene region, the sequence of the PuWB strain clustered with members classified in subgroups 16SrXII-J (GenBank accession number FJ409897) and 16SrXII-A (GenBank accession number AJ964960) (Figure 2a). On the tree of *tuf* genes, the PuWB strain clustered with '*Ca*. P. solani' strain PFY (GenBank accession number KC481242, 16SrXII-A) (Figure 2b).

Based on the virtual RFLP patterns generated using *i*Phyclassifier, the PuWB strain was different from the described representatives of subgroups 16SrXII-A to 16SrXII-K at the restriction site *Alu*I (Figure 3b). In addition, the phytoplasma had similarity coefficients ranging from 0.74 to 0.96 with the representatives of 16SrXII-A to 16SrXII-K subgroups (Supplementary table 1). These results indicated the possible occurrence of a novel subgroup in the 16SrXII group. Furthermore, the RFLP pattern generated using *Alu*I on the amplicons was identical to the virtual pattern (Figure 3a), confirming the reliability of virtual results. The PuWB phytoplasma is therefore considered a '*Ca*. P. solani'-related strain.

The phytoplasmas in the 16SrXII group are widely distributed, and the identification of the PuWB strain described here expands knowledge their genetic diversity. Phytoplasmas infecting *Cucurbita* spp. have been previously reported in several countries including Brazil, Japan, India, Iran, Egypt, Argentina, and have been

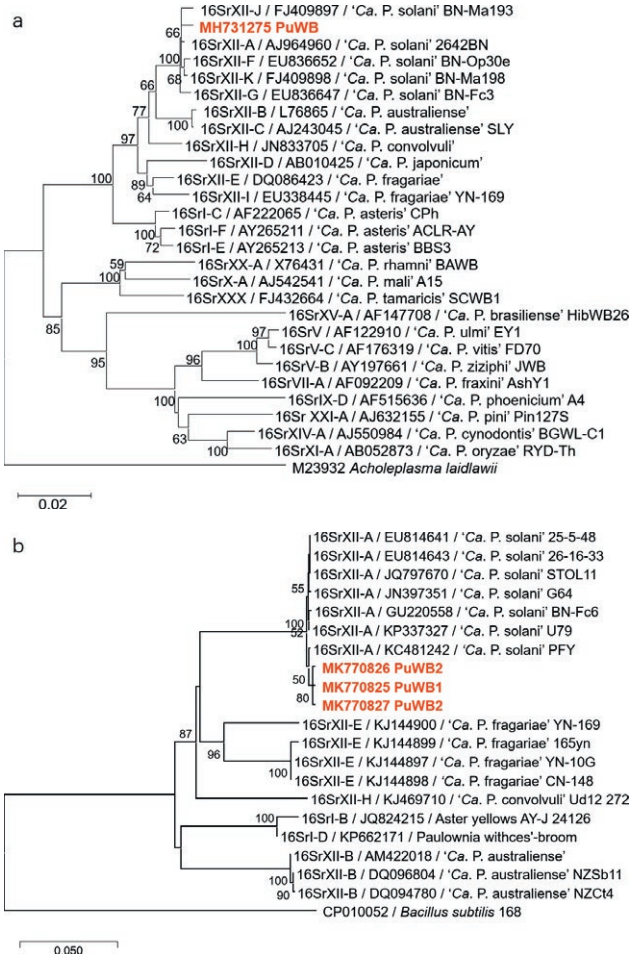

Figure 2. Phylogenies of PuWB phytoplasma strains and other phytoplasmas based on two conserved gene loci. The phylogenetic trees were reconstructed based on, a) the R16F2n/R2 regions, and b) the partial *tuf* genes of phytoplasmas. The sequences obtained in the present study are indicated in red font. Bootstrap values greater than 50% are shown. Bars = number of substitutions per base.

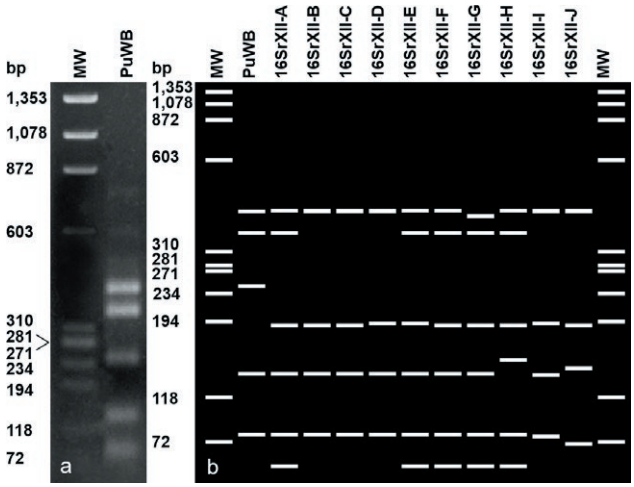

Figure 3. The RFLP patterns of the R16F2n/R2 region of the PuWB phytoplasma. a) PCR products digested using *Alu*I. b) *i*Phyclassifier generated virtual patterns of the PuWB phytoplasma and members of subgroups 16SrXII-A to -K. MW, φX174 DNA digested with *Hae*III.

associated with host symptoms of yellows, virescence, stunting, witches' broom and phyllody. The detected phytoplasmas belong to several ribosomal groups, including 16Sr I, -II and -III (Tanaka *et al.*, 2000; Montano *et al.*, 2006; Omar and Foissac, 2012; Galdeano *et al.*, 2013; Salehi *et al.*, 2015; Rao *et al.*, 2017). Identification of the PuWB strain in pumpkins showing witches' broom symptom in Xinjiang, China is the first record of a phytoplasma disease of pumpkin in China, and a first international record of a 16SrXII phytoplasma infecting pumpkin.

The occurrence of phytoplasmas in group 16SrXII has been reported previously in China, in *Artemisia scoparia* associated with witches' broom (Yu *et al.*, 2016) and *Peonia suffruticosa* associated with yellows symptoms in Shandong (Gao *et al.*, 2013), and *Salvia miltiorrhiza* with red-leaf symptoms in Shaanxi (Yang *et al.*, 2016). In addition, a phytoplasma in group 16SrII was detected in *Senna surattensis* stem fasciation (Wu *et al.*, 2012), and phytoplasmas related to '*Ca*. P. fragariae' were identified in *Solanum tuberosum* showing purplish leaves and aerial tuber formation (Cheng *et al.*, 2012).

The pumpkin plants exhibiting the symptom of witches' broom were mainly observed in Xinjiang, with incidence of approx. 1.5%. It is important, however, to record this disease in China for epidemiological reasons. Although the spread of PuWB was not fully evaluated, it is known that insects in *Auchenorrhyncha* are responsible for the transmission of phytoplasmas. These include members of *Cercopidae*, *Cixiidae*, *Derbidae*, *Delphacidae*, *Cicadellidae* and *Psyllidae* (Weintraub and Beanland, 2006). These insects were reported in Xinjiang, in particular leafhoppers (*Cicadellidae*) and psyllids (*Psyllidae*) (Zhang *et al.*, 2013; Chen *et al.*, 2018), and these insects are likely to disseminate PuWB phytoplasma-associated diseases, increasing the risk of severe epidemics and reductions of *C. moschata* production.

ACKNOWLEDGEMENT

This research was funded by the Research Start-up Funds for High-level Researchers in Inner Mongolia Agricultural University (Grant Numbers NDYB2018-3, NDYB2019-1), and the Natural Science Foundation of Inner Mongolia, China (Grant Numbers 2019MS03021).

COMPLIANCE WITH ETHICAL STANDARDS

Funding: This study was funded by the Research Start-up Funds for High-level Researchers in Inner Mongolia Agricultural University (Grant Numbers NDYB2018-3, NDYB2019-1), and the Natural Science Foundation of Inner Mongolia, China (Grant No. 2019MS03021).

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent: Informed consent was obtained from all individual participants included in the described research.

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