Short Notes

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

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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.

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 m²), 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.
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): CGC-CAGGGTTTTCCCAGTCAGGAC and M13 reverse primer (-48): AGCGGATAACAATTTCACAGGGA 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 16Srl 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 was submitted to the online tool Phyclassifier (https://plantpathology.ba.ars.usda.gov/cgi-bin/resource/iphyclasifier_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 AluI 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 tTufu/tTufu. 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 Phyclassifier, the PuWB strain was different from the described representatives of subgroups 16SrXII-A to 16SrXII-K at the restriction site AluI (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 AluI 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...
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 symp-

toms in Shandong (Gao et al., 2013), and Salvia milii-orrhiza 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, Delphaci- dae, Cicadellidae and Psyllidae (Weintraub and Beang-land, 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 epidem-

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COMPLIANCE WITH ETHICAL STANDARDS

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LITERATURE CITED

Bertaccini A., Lee I-M., 2018. Phytoplasmas: an update. In: Phytoplasmas: Plant Pathogenic Bacteria-I (G.P. Rao, A. Bertaccini, N. Fiore, L.W. Liefting, eds.), Springer, Singapore, 1–29.

Bertaccini A., 2019. The phytoplasma classification between ‘Candidatus species’ provisional status and ribosomal grouping system. Phytopathogenic Molliscutes 9: 1-2.

Chen P., Liu Q-Z., Qiao X-F., Wang J-W., Zhang T., 2018. Identification and phylogenetic analysis of pear psyl lids (Hemiptera: Psyllidae) in Chinese pear orchards. Journal of Economic Entomology 111: 2908–2913.

Cheng M., Dong J., Zhang L., Laski P., Zhang Z., McBeath J., 2012. Molecular characterization of stolbur group subgroup E (16SrXII-E) phytoplasma associated with potatoes in China. Plant Disease 96: 1372–1372.

Cheng C., Liang X., Xiang B., Zheng Y., 2013. Analysis of Cucumber mosaic virus satellite RNA and RNA3 sequence of processing tomato and pumpkin in Shilhezi of Xinjiang. Acta Agriculturae Boreali-Occidentalis Sinica 5: 83–90.

Contaldo N., Bertaccini A., Paltonieri S., Windsor H.M., Windsor G.D., 2012. Axenic culture of plant pathogenic phytoplasmas. Phytopathologia Mediterranea 51: 607–617.

Contaldo N., Satta E., Zambon Y., Paltrinieri S., Bertaccini A., 2016. Development and evaluation of different complex media for phytoplasma isolation and growth. Journal of Microbiological Methods 127: 105–110.

Contaldo N., Bertaccini A., 2019. Phytoplasma Cultivation. In: Phytoplasmas: Plant Pathogenic Bacteria-III (A. Bertaccini, K. Oshima, M. Kube, G. Rao, eds.), Springer, Singapore, 89–104.

Deng S., Hiruki C., 1991. Genetic relatedness between two nonculturable mycoplasmalike organisms revealed by nucleic acid hybridization and polymerase chain reaction. Phytopathology 81: 1475–1479.

Devi N., Prasad R., Sagarika N., 2018. A review on health benefits and nutritional composition of pumpkin seeds. International Journal of Chemical Studies 6: 1154–1157.

Doi Y., Teranaka M., Yora K., Asuyama H., 1967. Mycoplasma- or PLT Group-like microorganisms found in the phloem elements of plants infected with mulberry dwarf, potato witches’ broom, aster yellows, or paulownia witches’ broom. Japanese Journal of Phytopathology 33: 259–266.

Galdeano E., Guzmán F.A., Fernández F., Conci L.R., 2013. Genetic diversity of 16SrIII group phytoplasmas in Argentina. Predominance of subgroups 16SrII-J and B and two new subgroups 16SrIII-W and X. European Journal of Plant Pathology 137: 753–764.

Gao Y., Qiu P.P., Liu W.H., Su W.M., Gai S.P., Liang Y.C., Zhu X.P., 2013. Identification of ‘Candidatus Phytoplasma solani’ associated with tree peony yellows disease in China. Journal of Phytopathology 161: 197–200.

Gundersen D.E., Lee I-M., 1996. Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. Phytopathologia Mediterranea 35: 144–151.

Hogenhout S.A., Segura M., 2010. Phytoplasma genomics, from sequencing to comparative and functional genomics—What have we learnt. In: Phytoplasmas: Genomes, Plant Hosts Vectors (P.G. Weintraub, P. Jones, eds.), CAB International, Wallingford, UK, 19–36.

IRPCM, 2004. ‘Candidatus Phytoplasma,’ a taxon for the wall-less, non-helical prokaryotes that colonize plant phloem and insects. International Journal of Systematic Evolutionary Microbiology 54: 1243–1255.

Kollar A., Seemüller E., Bonnet F., Saillard C., Bové J-M., 1990. Isolation of the DNA of various plant pathogenic mycoplasmalike organisms from infected plants. Phytopathology 80: 233–237.

Kumar V., Mishra D., Yadav G., Yadav S., Kumar S., 2018. Determining relationships between yield and biochemical traits in pumpkin. The Pharma Innovation Journal 7: 14–18.

Lee I-M., Gundersen-Rindal D.E., Davis R.E., Bartoszyk I.M., 1998. Revised classification scheme of phytoplasmas based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences. International Journal of Systematic Evolutionary Microbiology 48: 1153–1169.
Montano H., Brioso P., Pimentel J., Figueiredo D., Cunha Junior J., 2006. *Cucurbita moschata*, new phytoplasma host in Brazil. *Journal of Plant Pathology* 88: 226.

Nejat N., Sijam K., Abdullah S.N.A., Vadomalai G., Dickinson M., 2010. Molecular characterization of an aster yellows phytoplasma associated with proliferation of periwinkle in Malaysia. *African Journal of Biotechnology* 9: 2305–2315.

Omar A.F., Foissac X., 2012. Occurrence and incidence of phytoplasmas of the 16SrII-D subgroup on solanaceous and cucurbit crops in Egypt. *European Journal of Plant Pathology* 133: 353–360.

Peng B., Kang B., Wu H., Liu L., Liu L., Fei Z., ... Gu Q., 2019. Detection and genome characterization of a novel member of the genus *Polerovirus* from zucchini (*Cucurbita pepo*) in China. *Archives of Virology* 164: 2187–2191.

Quanhong L., Caili F., 2005. Application of response surface methodology for extraction optimization of germinant pumpkin seeds protein. *Food Chemistry* 92: 701–706.

Rao G., Gopala G.S., Rao A., 2017. First report of a *Candidatus Phytoplasma asteris*-related strain (16SrI-B subgroup) associated with witches’ broom disease in *Cucurbita pepo* in India. *New Disease Reports* 35: 33.

Salehi M., Siampour M., Esmaizadeh Hosseini S., Bertacini A., 2015. Characterization and vector identification of phytoplasmas associated with cucumber and squash phyllody in Iran. *Bulletin of Insectology* 68: 311–319.

Schneider B., Seemüller E., Smart C., Kirkpatrick B., 1995. Phylogenetic classification of plant pathogenic mycoplasma-like organisms or phytoplasmas. In: *Molecular and diagnostic procedures in Mycoplasmology* (S. Razin, J. Tully, ed.), Academic Press, New York, USA, 369–380.

Schneider B., Gibb K.S., Seemüller E., 1997. Sequence and RFLP analysis of the elongation factor Tu gene used in differentiation and classification of phytoplasmas. *Microbiology* 143: 3381–3389.

Tanaka M., Osada S., Matsuda I., 2000. Transmission of rhus (*Rhus javanica*) yellows by *Hishimonus sellatus* and host range of the causal phytoplasma. *Journal of General Plant Pathology* 66: 323–326.

Weisburg W., Tully J., Rose D., Petzel J., Oyaizu H., Yang D., ... van Etten J., 1989. A phylogenetic analysis of the mycoplasmas: basis for their classification. *Journal of Bacteriology* 171: 6455–6467.

Yang R., Wang G., Wang S., Zhang D., Wei L., Chen H., ... Hu X., 2016. Molecular identification and diversity of *Candidatus Phytoplasma solani*’ associated with red-leaf disease of *Salvia miltiorrhiza* in China. *Journal of Phytopathology* 164: 882–889.

Yu X., Ai C., Wang J., Fu L., An M., Wang H., Sun Q., 2016. First report of a 16SrXII-A subgroup phytoplasma associated with *Artemisia scoparia* witches’ broom disease in China. *Plant Disease* 100: 1494–1494.

Zhao Y., Lu L., Yong K.J., 2013. Review of the leafhopper genus *Macrosteles* Fieber (Hemiptera: Cicadellidae: Deltocephalinae) from China. *Zootaxa* 3700: 361–392.

Zhao Y., Davis R.E., 2016. Criteria for phytoplasma 16Sr group/subgroup delineation and the need of a platform for proper registration of new groups and subgroups. *International Journal of Systematic Evolutionary Microbiology* 66: 2121–2123.