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Molecular identification of a Candidatus phytoplasma (Group16SrV-D) coding partial uvrB gene and degV gene on a new host – mesta (*Hibiscus sabdariffa*) – with phyllody and reddening of leaves in India

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Abstract Mesta (*Hibiscus sabdariffa*) is an important bast fiber crop. In August 2011, there was an outbreak of a phytoplasma-like disease on *H. sabdariffa* in different villages of the northern coastal mesta-growing region of Andhra Pradesh, India, covering mainly two districts – Srikakulam and Vijayanagaram. The infected plants showed characteristic symptoms such as phyllody and reddening of leaves. PCR with P1/P7 universal primer pair of 16 S rDNA yielded amplicons of 1850 bp from all symptomatic mesta leaf samples similar to samples of brinjal little leaf (phytoplasma positive reference control). However, asymptomatic samples were not amplified. Multiplex nested-PCR showed simultaneous amplification of DNA fragments with phytoplasma specific primers, *viz.,* P1/P7 universal primer pair of 16 S rDNA, nested primer pair R16F2n/R2, uvrB and DegV gene-specific uvrB-degVF/R primer generating amplicons of 1850 bp, 1200 bp and 1023bp, respectively. However, SecY-map gene specific primer SecY-mapF/R was not amplified. The 1023 bp nucleotide sequence of uvrB and DegV gene of the phytoplasma was deposited in the GenBank (NCBI) with the accession no. JX975061. NCBI BLASTn analysis of the 1023 bp products showed that the phytoplasma strain belonged to elm yellows group (16SrV-D). This is the first report that *Hibiscus sabdariffa* is infected by a phytoplasma and we named it mesta phyllody disease (MPD).

Keywords Nested PCR · Roselle · 16SrV · Sequence analysis

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Mesta or roselle (*Hibiscus sabdariffa* Linn.) is widely grown in the tropics of Central America, India, Africa, Brazil, Australia, Hawaii, Florida, Caribbean islands and Philippines (Mahadevan *et al.* 2009). It has valuable industrial use because of its bast fiber, medicinal value and application in the paper industry (Chatterjee *et al.* 2005). In India, mesta is cultivated in various parts of Andhra Pradesh, West Bengal, Madhya Pradesh, Maharashtra, Bihar, Orissa, Assam, Punjab and Uttar Pradesh during April to November (Mahadevan *et al.* 2009). The crop suffers from many diseases such as foot and stem rot, yellow vein mosaic, etc. (Biswas *et al.* 2011). But phytoplasma diseases are not known to affect this crop. In August 2011, there was an outbreak of a phytoplasma-like disease on *H. sabdariffa* in different villages of the northern coastal mesta-growing region of Andhra Pradesh, India, covering mainly two districts, Srikakulam and Vijayanagaram. Approximately 5000

ha of cropped area was affected and the disease incidence varied from 13% to 32%. The infected plants showed characteristic symptoms such as phyllody and reddening of leaves (Fig. 1a, b). The mature lower leaves were red in color, but in younger leaves only the margins were red. The newly emerged tender leaves turned narrower and smaller, clustering at the apical region. Some leaves were also deformed. There was proliferation of thick, small and succulent auxiliary branches. White hairy structures were present on tender leaves and branches. The dwarfing of plants was also common, with conspicuous shortening and thickening of internodes. In the present study we tried to ascertain whether the said disease in mesta was caused by a phytoplasma through PCR amplification of 16S rDNA and, if so, to identify the phytoplasma group based on phylogeny.

Leaf samples were taken from naturally infected mesta (*H. sabdariffa*) plants collected from farmers' fields of Amadalavalasa in the Srikakulam district of Andhra Pradesh, India. Six symptomatic plants suggestive of phytoplasma infection and six asymptomatic healthy looking plants were randomly selected for collecting the samples. DNA extraction technique was standardized to obtain both plant and phytoplasmal r-DNA from infected samples. Approximately 300 mg of leaf tissue was taken from each sample. An uninfected healthy sample served as control. A sample of brinjal little leaf was taken as a phytoplasma positive reference control. DNA was extracted from all the samples by using the CTAB method (Kollar *et al.* 1990).

All the leaf samples of both symptomatic as well as asymptomatic *H. sabdariffa* plants and brinjal little leaf sample (positive reference control) were assessed by PCR using a phytoplasma specific primer. Phytoplasma-specific universal primers P1/P7 (Deng & Hiruki 1991) were used for PCR amplification. To test the presence of phytoplasma, purified rDNA (6 μl with a concentration of 10 ng μl^{-1}) was used for PCR amplification. The amplification was carried out in a thermal cycler (Bio-Rad T100, Bio-Rad Laboratories Inc., Hercules, CA, USA) with 30 μl reaction mixture consisting of 0.5 μM primers, 200 μ mol l^{-1} dNTPs (New England Biolabs, Ipswich, MA, USA), 25 m mol l^{-1} $MgCl_2$ (Biolabs), 3U Taq DNA polymerase (Biolabs) and 10x PCR buffer (Biolabs). The PCR conditions employed were denaturation at 94°C for 5 min, 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, followed by 30 cycles and final extension at 72°C for 10 min. The product was diluted in a ratio of 1:30 and 1 μl of it was used in nested-PCR as the template with primer pair R16F2n/R2 (Gundersen & Lee 1996), which covers 1850 bp of the 16S rDNA region. The PCR profile was the same as with universal primers and the only variation was in the annealing temperature (56°C). The amplified products were analyzed by electrophoresing on 1.6% agarose gel in Tris-Acetate EDTA, *i.e.,* TAE (0.45 M Tris (pH 8.0), 0.5 M EDTA (pH 8.0), and 0.8 M glacial acetic acid) containing ethidium bromide (25 mg ml^{-1}).

Fig. 1 (**a**) Mesta plants showing symptoms of phyllody and reddening of leaf margins. (**b**) Mesta plant with proliferation of apical branches and red leaves

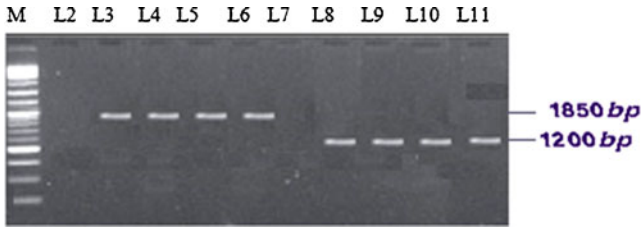

Fig. 2 Agarose gel showing bands of 1850 bp amplified by direct PCR with P1/P7 primers from symptomatic mesta samples (lanes 3–5) and ~1200 bp bands by nested PCR with R16F2n/R16R2 primers (lanes 8–10). Lanes 2 and 7: healthy control from asymptomatic samples; lanes 6 and 11: phytoplasma positive reference control (little leaf of brinjal) amplified by P1/P7 and nested primers, respectively. M: 1kb ladder

A multiplex PCR assay was standardized for simultaneous amplification of photoplasma DNA by using multiple primers, *viz.,* P1/P7 (Deng & Hiruki 1991) universal primer pair, nested primer pair R16F2n/R2 (Gundersen & Lee 1996), uvrB –degV gene specific primer uvrB-degVF/R and map gene specific primer SecY-map F/R (Malembic-Maher *et al.* 2011). Concentrations and quantities of different PCR components were optimized. The concentrations of $MgCl_2$, primer pairs, dNTPs, 10 x PCR buffer and Taq DNA polymerase (3U μl^{-1}) and rDNA (100 ng μl^{-1}) were increased. The amount of nuclease-free water and the concentration of primers (100 μ mol l^{-1}) were also standardized. The PCR master mix (50 μl) contained 5 μl of 10 X PCR buffer (Biolabs), 3 μl of 25 m mol l^{-1} $MgCl_2$ (Biolabs), 2 μl of 100 μ mol l^{-1} dNTPs (Biolabs), 0.5 μl of 100 μ mol l^{-1} of each forward and reverse primer, 1 μl of Taq DNA polymerase (3U μl^{-1}) and 30 μl of nuclease-free water. Before adding Taq polymerase, other reagents were mixed and the mixture was subjected to 94°C for 5 min for an initial denaturation. Variable numbers of programming cycles were employed to ensure amplification of all the three primers and also to overcome false amplifications. Taq polymerase was added to the reaction mixture after hot-start. The amplification profile was 2 min initial denaturation at 94°C, 10 cycles of denaturation at 94°C for 15 s, annealing temperature at 54.5°C for 30 s, followed by 20 cycles of denaturation at 94°C for 30 s, annealing at 54.5°C for 30 s, elongation at 72°C for 45 s and additional elongation at 72°C for 7 min.

PCR with P1/P7 universal primer pair of 16 S rDNA yielded amplicons of 1850 bp from all symptomatic mesta leaf samples similar to samples of brinjal little leaf (phytoplasma positive reference control). However, asymptomatic samples were not amplified (Fig. 2). Multiplex nested-PCR showed simultaneous amplification of DNA fragments with phytoplasma specific primers, *viz.,* P1/P7 universal primer pair of 16 S rDNA, nested primer pair R16F2n/R2, uvrB and DegV gene-specific uvrB-degVF/R primer generating amplicons of 1850 bp, 1200 bp and 1023bp, respectively. However, SecY-map gene-specific primer SecY-mapF/R was not amplified (Fig. 3).

Five symptomatic PCR (P1/P7) positive samples were sequenced and they showed 100% similarity with one another. The isolate MPH-HS-11(1850 bp) showed 99.9% identity with partial 16S ribosomal RNA gene (Accession Nos. HQ712064, AF176319 and HQ712065). As SecY-map gene specific primer did not show any amplification, the 1023 bp amplicons generated by uvrB-degVF/R primer from infected samples were eluted through PCR clean-up system (Promega, Madison, WI, USA) and cloned. The clones were sequenced. One of them was deposited in GenBank with Accession no. JX975061). The NCBI BLASTn program was used to scan the sequence data against other phytoplasma 16S rRNA gene sequences. A phylogenetic tree was constructed by the neighbor joining method using the MEGA software version 4 (Saitou & Nei 1987; Tamura *et al.* 2007). The BLASTn analysis of the nucleotide sequence (1023 bp) revealed that the present mesta phytoplasma isolate MPH-HS-11 had

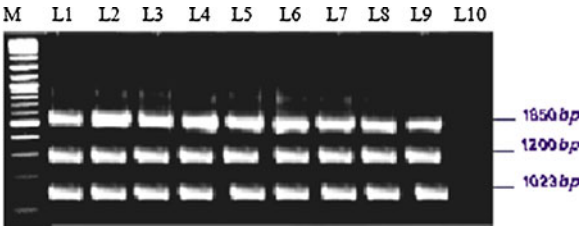

Fig. 3 Multiplex nested-PCR amplification by universal P1/P7 primers, R16F2n/R16R2 nested PCR primers, uvrB-degV gene-specific primer and secY-map gene-specific primer from symptomatic samples (lanes 1–9); lane 10: asymptomatic sample. M: 1kb ladder

100% homology with Candidatus phytoplasma vitis partial uvrB gene for excinuclease ABC subunit B and partial degV gene for DegV family protein (Acc. FN811143, Acc. AM396422, Acc. AM396414). MPH-HS-11 isolate was found to cluster with the 16SrV-D group (Fig. 4). Sequence analysis indicated that this isolate had two protein-coding regions. Using nucleotide span and ORF finder (NCBI) analysis data, BioEdit translated amino acid sequences of the concerned two proteins. Protein BLAST analysis revealed that of the two translated protein sequences, one had 100% similarity with Protein ID: CBL59894 and the other had 100% similarity with Protein ID: CBL59895. Domain prediction of the translated protein revealed the presence of conserved domain partial uvrB gene for excinuclease ABC subunit B (InterProScan id: I PR001943) and partial Deg V gene for Deg V family protein (IPR 0037970).

PCR amplification and phylogenetic analysis showed that the present mesta disease was caused by a phytoplasma belonging to the 16SrV-D subgroup. Elm yellows group (16 Sr V) phytoplasmas, which are generally associated with widespread diseases in elm, grapevine, blackberry, cherry, peach and several other plant species in America, Europe and Asia, represent the third largest phytoplasma cluster after yellows and X disease (Lee *et al.* 2004). 'Candidatus P. ulmi' in the 16SrV-A subgroup is responsible for yellows of elm species in North America and Europe (Lee *et al.* 2004); 'Candidatus P. ziziphi' in the 16SrV-B subgroup is the agent of jujube witches'-broom and cherry lethal yellows in Asia (Jung *et al.* 2003; Lee *et al.* 2004). Other phytoplasmas in the 16SrV group are reported to infect mainly grapevines (Maixner *et al.* 1994), alder (Lederer & Seemüller 1991; Mäurer *et al.* 1993), blackberry (de Fluiter & van der Meer 1953; Mäurer & Seemüller 1995), species of the genus Spartium (Marcone *et al*. 1996) and *Clematis vitalba* (Angelini *et al.* 2004) in Europe. PGY and AldY phytoplasmas are classified as members of the 16SrV group on the basis of their high 16S rRNA and secY gene sequence similarities to the corresponding genes of phytoplasmas (Angelini *et al.* 2001, 2003). Phytoplasmas have been classified into two distinct subgroups, 16SrV-C and 16SrV-D, on the basis of sequence differences in the 16S rRNA gene and the 16S–23S intergenic spacer (Davis & Dally 2001; Martini *et al.* 1999). Malembic-Maher *et al.* (2008) reported that 16SrV group phytoplasmas showed significant molecular diversity at the genome level.

Peach yellows phytoplasma and jujube witches' broom phytoplasma both belonging to the subgroup 16 Sr V-B are the major members of elm yellows group (16 Sr V) reported from India (Lee *et al.* 2004). The present phytoplasma isolate MPH-HS-11, which caused phyllody and reddening of leaves on mesta in an epidemic form, showed 100% homology with 16Sr V-D (uvrB and deg V locus). To the best of our knowledge it is the first report of a phytoplasma of 16 Sr V-D sub-group from India. We

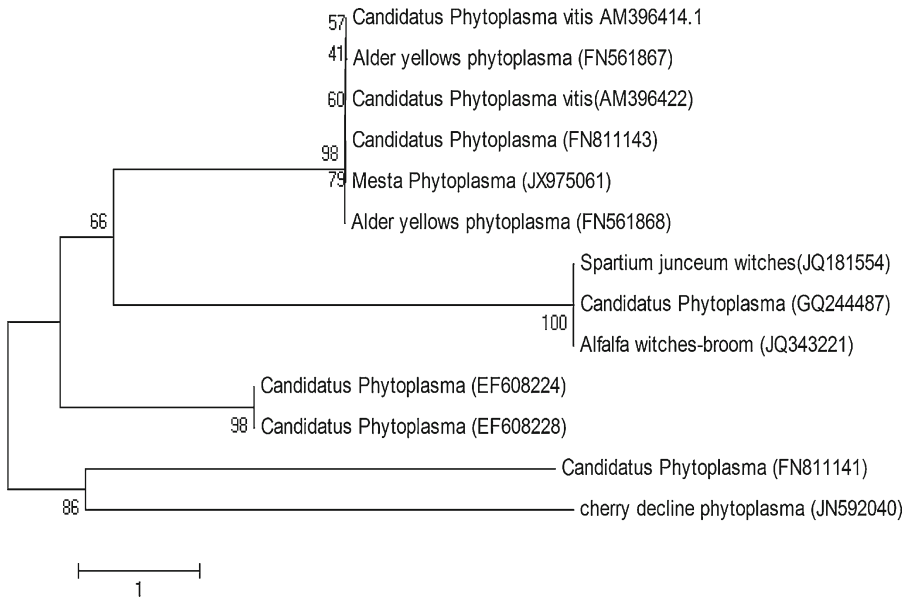

Fig. 4 Phylogenetic tree constructed using the maximum parsimony method of MEGA 4.1 for a uvrB –degV gene and 12 other reference phytoplasmal sequences retrieved from GenBank. Bootstrap values are noted at each node

have named the disease mesta phyllody disease (MPD). It is also the first report that *Hibiscus sabdariffa* (mesta) is infected by a phytoplasma.

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