First report of '*Candidatus Phytoplasma aurantifolia*' (16SrII-A) associated with strawflower phyllody

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Received: 21 March 2019 / Accepted: 1 August 2019 / Published online: 7 August 2019

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Abstract

Four strawflower plant samples showing phyllody disease symptoms were collected from the experimental plot at the College of Horticulture, Bangalore, Karnataka (India). The phytoplasma infection in strawflower was confirmed through PCR using 16Sr RNA and SecY gene specific primers. All four strawflower plant samples gave positive amplification 16Sr RNA (1.8 kb) and SecY gene (1.6 kb) for the phytoplasma. The amplified products were cloned and sequenced. The sequences of four clones obtained were identical. Therefore, a representative isolate (SWF1) nucleotide (nt) sequence of 16Sr RNA and SecY gene comparisons were made with the available phytoplasmas 16Sr RNA and SecY gene nt sequences from NCBI database. The analysis showed that strawflower phyllody phytoplasma shared the highest identity of 97.3 to 98.5% (16S rRNA) and 99.4 to 99.5% (SecY gene) with '*Candidatus Phytoplasma aurantifolia*' group (16SrII) isolates reported from different parts of the world. This was well supported by close clustering of strawflower phytoplasma in the current study with '*Ca. P. aurantifolia*' 16SrII groups in the phylogenetic analysis. The virtual RFLP pattern generated for the phytoplasma from strawflower phyllody was similar (similarity coefficient 0.97) to the reference pattern of 16Sr group II and subgroup A ('*Ca. P. aurantifolia*', 16SrII). This is the first report of phytoplasma associated with the phyllody disease of strawflower in India.

Keywords Witches broom disease · Phytoplasma · PCR · Phylogenetic analysis · '*Ca. P. aurantifolia*' · 16SrII

Strawflower (*Helichrysum bracteatum*) is an important ornamental plant belonging to family Asteraceae, native to Australia. The strawflower is very popular worldwide for dry bouquets due to durability of its flowers and it is widely cultivated as an ornamental garden plant (Nishikawa et al.

2008; Morley 1978). The plants are upright warm-weather annuals or short lived perennials with daisy-like flower heads in yellow, pink, bronze, cream, purple or white colours. They are commonly used for dried floral arrangements, due to longevity even after dry without losing color and shape. This economic ornamental plant is highly susceptible to many diseases is major limiting factor for its cultivation in different parts of world. The important diseases recorded on strawflower are *Sclerotinia sclerotiorum* (Duarte and Barreto 2009; Boland and Hall 1994; Bolton et al. 2006; Jun and Hiromichi 1999), Downy mildew (Garibaldi et al. 2003), *Botrytis cinerea* (Garibaldi et al. 2017) and Aster yellow (16SrI) phytoplasma (Franov et al. 2003). In the present study we have characterised another phytoplasma belonging to '*Candidatus Phytoplasma aurantifolia*' (16SrII-A) associated with strawflower phyllody in Karnataka, India.

In June 2017, typical symptoms of phytoplasma disease, including phyllody and witches'-broom were observed on strawflowers (*Helichrysum bracteatum*) planted in the experimental plot at the College of Horticulture (77.5 °E longitude; 13.07 °N latitude), Bangalore, Karnataka (India). One leaf sample without any symptoms was also collected to be used as a negative control. The identity of the phytoplasma

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s13314-019-0359-0>) contains supplementary material, which is available to authorized users.

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Fig. 1 Strawflower plants are showing phyllody symptoms under natural conditions

associated with the strawflower phyllody was confirmed by PCR detection. For PCR detection, total genomic DNA was extracted separately from flowers and leaves of five symptom-bearing and one symptomless plant using Cetyl trimethyl ammonium bromide (CTAB) method as described by Doyle and Doyle (1990). The DNA isolated from a plant infected with a known phytoplasma (Sesame and brinjal) was used as a positive control and from samples without symptoms as negative controls. Both direct and nested PCR assays were performed using phytoplasma universal 16S rDNA primer pairs P1/P7 (Deng and Hiruki 1991; Smart et al. 1996), R16F2n/R16R2 (Gundersen and Lee 1996) and SecY gene primers pairs SecYF2 (II) and SecYR1 (II) as described by Lee et al. (2010). The resulting PCR amplicons (1.8 kb) and nested PCR amplicons of 1.2 kb corresponding to the phytoplasma 16S ribosomal RNA and 1.6 kb of SecY gene were amplified in all four strawflower and positive samples. There was no amplification in the samples collected from non-symptomatic plants. The amplified PCR products of P1/P7 primers (1.8 kb) and SecYF2/R1 (1.6 kb) derived from four strawflower plant samples were cloned into pTZ57R/T cloning vector according to manufacturer's directions (MBI Fermentas, Germany). Recombinant clones were identified by PCR as well as restriction endonuclease digestion and clones were sequenced. The sequences of the four clones obtained were identical. Therefore, a representative sequence (SWF1 phyllody phytoplasma) of 16SrRNA (accession number MF996473) and SecY gene (accession number MF996474) were deposited in GenBank respectively (Fig. 1).

The nucleotide sequence of 16S rRNA and SecY gene of SWF1 phyllody phytoplasma were checked (Table S1) and aligned using SEAVIEW program (Galtier et al. 1996) and

ClustalX softwares. The phylogenetic tree was generated by MEGA 7 software (Kumar et al. 2016) using the neighbour joining method with 1000 bootstrapped replications to estimate evolutionary distances between all pairs of sequences simultaneously. *In-silico* RFLP patterns were generated from the phytoplasma 16S ribosomal sequence using the gel plotting program pDRAW32 (<http://www.acaclone.com/>) and iPhyClassifier software (Zhao et al. 2009).

The SWF1 phyllody phytoplasma 16SrRNA (Acc.No. MF996473) and SecY gene (Acc.No. MF996474) sequence from Karnataka obtained in the current study was compared with sixty one 16S rRNA and fifty six SecY gene sequences of different groups of phytoplasma that were retrieved from NCBI database were used in the comprehensive analyses (Table S1). The analysis of 16SrRNA sequence showed that the phytoplasma associated with strawflower phyllody shared nucleotide identity from 97.3 to 98.5% with '*Ca. P. aurantifolia*' -16SrII [KX008308, KX008310] and the SecY gene shared 99.4 to 99.5% with Tomato big bud (KT970083, KT970081, KC953016, KT970084, KT970082, KT970080, KT970078) and Cauliflower phyllody [KC953012], Sword bean witches-broom [KC953015], Parthenium virescence [KC953014] all belonging to '*Ca. P. aurantifolia*' (16SrII group).

The phylogenetic neighbour-joining tree was constructed (MEGA 7 software) based on 16SrRNA (Acc.No. MF996473) and SecY gene (Acc.No. MF996474) sequence of SWF1 phyllody with corresponding region 16SrRNA and SecY gene sequences of different groups of phytoplasma retrieved from database (Table S1). The results showed that the strawflower phyllody 16SrRNA and SecY gene clustered with '*Ca. P. aurantifolia*' related strain (Fig. 2). The analysis

Fig. 2 Phylogenetic tree based on sequences of 16S rRNA (a) and SecY gene (b) of Strawberry phyllody phytoplasma (SWF1) with other phytoplasma strains using Neighbor-joining algorithm. Horizontal distances are

proportional to sequence distances, vertical distances are arbitrary. The trees are unrooted. A bootstrap analysis with 1000 replicates was performed and the bootstrap percent values more than 50 are numbered along branches

showed that the Indian strawberry infecting phytoplasmas formed a monophyletic cluster with Asian-Australasian-Saudi Arabia origin phytoplasmas and established the close

relationship with 16SrII-A. The analysis also showed that the oligonucleotide sequences complementary to unique regions of the 16S rRNA 5'-TAAAGGCATCTTTTATC- 3' and 5'-

CAAGGAAGA AAA GCAAATGGCGAACCATTGTTT-3' of strawflower phyllody phytoplasma was similar to the 16SrII 'Ca. P. aurantifolia' related strain.

In-silico RFLP analysis of F2nR2 fragment of 16SrRNA sequence of SWF1 phyllody phytoplasma by using online tool iPhyClassifier (<https://plantpathology.ba.ars.usda.gov/cgi-bin/resource/iphyclassifier.cgi>) indicated that the virtual RFLP pattern derived from the query of F2nR2 fragment of 16SrRNA sequence of strawflower phyllody phytoplasma had similarity coefficient of 0.97 in reference to pattern of 16Sr group II and subgroup A (Gen Bank accession: L33765, 'Ca. P. aurantifolia' -16SrII). Similarity coefficients of 16S rRNA gene RFLP patterns were used in classifying phytoplasma into groups and sub groups with the threshold similarity coefficient for new subgroup delineation set at 0.97 (Lee et al. 1998; Wei et al. 2007). A strain is treated as new if a phytoplasma strain's virtual 16S rRNA gene RFLP pattern similarity coefficient is less than or equal to 0.97 with those of all existing representative strains of the given group. Therefore, the phytoplasma identified in the present study is belongs to *Ca. P. aurantifolia* (16SrII) group and subgroup A. This is the first report of 16SrII phytoplasma affecting Strawflower in India.

Phytoplasmas belong to genus mollicutes infecting several plant species (Al-Saady and Khan 2006; Harrison et al. 2008) and cause serious economic losses in many ornamental plants (Chaturvedi et al. 2010). In the present study, the phytoplasma characterised from strawflower that belongs to 'Ca. Phytoplasma aurantifolia' -related strain (16SrII). which is associated with many diseases such as witches broom disease of lime, cabbage yellow, clover little leaf, alfalfa witches broom, tomato witches broom, sunflower phyllody, cucumber and squash phyllody, carrot witches broom, parsley witches broom, pomegranate little leaf, Phyllody of China Aster, mung bean phyllody and bell pepper bigbud in different parts of the world (Ragimekula et al. 2014; Mahadevakumar et al. 2017; Hemmati and Nikooei 2017). The result also showed that 'Ca. P. aurantifolia' -16SrII is expanding its host range by infecting different crops throughout the world.

The phytoplasma associated with strawflower were previously reported by Franov et al. (2003), who demonstrated that Aster yellow (16SrI) phytoplasma belonging to *Ca. P. asteris* related strains could infect this species in the Czech Republic. However, to our knowledge, this is the first report of an association of *Ca. P. aurantifolia*-related strain with strawflower in India.

Acknowledgements The research was supported by project on "Consortium platform on Vaccines and diagnostics", Indian Council of Agricultural Research, Government of India, New Delhi, India.

Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

Ethical approval This article does not contain any studies related to human or animal subjects performed by any of the authors.

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