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Multilocus sequence analysis of *Candidatus* Phytoplasma aurantifolia associated with phyllody disease of gerbera from India

Kannappanahalli Venkatareddy Ashwathappa[1], Venkataravanappa Venkataravanappa[2*], Lakshminarayana Reddy Cheegatagere Narasimha Reddy[3*], Krishna Reddy Manem[1]

[1]ICAR-Indian Institute of Horticultural Research, Hessaraghatta Lake PO, Bangalore-560089, Karnataka, India; [2]CHES, ICAR-Indian Institute of Horticultural Research Chettalli, Madikeri District-571248, Karnataka, India; [3]Department of Plant Pathology, College of Agriculture, University of Agricultural Sciences, GKVK, Bangalore-560065, Karnataka, India

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Summary. – Gerbera is the most popular cut flower known for its variety of colors and is grown across the world. Its production is challenged by numerous diseases affecting production and quality. During our survey, ten samples from the gerbera plants exhibiting phyllody disease symptoms were collected from Bangalore Rural District, Karnataka, India. The association of phytoplasma with the gerbera phyllody samples was confirmed by PCR using 16SrRNA, *Sec*Y, Ribosomal protein (*rp*) and *Sec*A gene-specific primers. PCR products were amplified from all ten gerbera plants using phytoplasma-specific primers. The amplified PCR products were cloned and sequenced; the sequences of the ten clones were identical. Therefore, representative isolate (GePP1, Gerbera phyllody phytoplasma) was selected for further analysis. The sequence analysis showed that GePP1 shared maximum nucleotide (nt) identity of 97.1% (16SrRNA) with Eggplant big bud, 98.7 to 98.8% (*Sec*Y gene) with Tomato big bud, 99.2 to 99.6% (*rp* gene) with Alfalfa witches-broom (EF193371) and 99.1% (*Sec*A gene) with Sesame phyllody phytoplasmas and that it belongs to the *Ca*. P. aurantifolia (16SrII) group. This result was well supported by the phylogenetic analysis showing GePP1 (16Sr RNA, *Sec*Y, *rp* and *Sec*A genes) closely clustering with the *Ca*. P. aurantifolia 16SrII group isolates reported so far. The virtual RFLP pattern generated for the phytoplasma from gerbera was different (similarity coefficient 0.89) from the reference pattern of *Ca*. P. aurantifolia (16Sr II) subgroup after analysis with four enzymes (*Bfa*I, *Hha*1, *Sau3A*I and *Rsa*I). Based on the threshold similarity coefficient for a new subgroup (delineation should be set at 0.97), the GePP1 may be considered as new subgroup of *Ca*. P. aurantifolia (16SrII) group. This is the first report of *Ca*. P. aurantifolia belonging to 16Sr II group affecting gerbera in India.

Keywords: Candidatus Phytoplasma aurantifolia; phyllody; gerbera; PCR; phylogenetic analysis

Introduction

Gerbera (*Gerbera jamesonii* Bolus ex. Hook f.), also known as "African Daisy" or Transvaal daisy, Barberton daisy or Verdt daisy flower, belongs to the family *Asteraceae* and is one of the most popular flowers among florists (Parthasarathy and Nagaraju, 1999). Gerbera is a perennial herb native to South Africa and Asia (Kanwar and Kumar, 2008) and grown throughout the world under a wide range of climatic conditions. Due to beautiful colors, hardy nature, long-keeping quality and ability to rehydrate after long transportation, gerbera has been considered suitable for export as well as domestic markets of India (Aswath and Rao, 2006). The production of gerbera was approximately worth US $ 220 million in 2001 and US $ 70 million was from stems sold in the US alone (Broek *et*

*Corresponding authors. E-mail: V Venkataravanappa@icar.gov.in, venkatrajani@gmail.com, phone:+9108276-266635 (V. Venkataravanappa); E-mail: cnlreddy@gmail.com, phone: +91-80-23330153 extn.294 (R. C. N. Lakshminarayana Reddy).

Abbreviations: *Ca* P. aurantifolia = *Candidatus* Phytoplasma aurantifolia; GePP1 = Gerbera phyllody phytoplasma; nt = nucleotide; RFLP = restricted fragment length polymorphism; SecA = protein translocase subunit; SecY = protein translocase subunit

al., 2004). The major constraints for the cultivation of gerbera are fungal, bacterial, viral and phytoplasma diseases resulting in reduced numbers and commercial quality of flowers. In India, *Ca*. P. asteris phytoplasma was reported to infect gerbera with 15–20% incidence; infected plants showing symptoms such as severe leaf yellowing, stunting and flower deformation (Siddique, 2005, Gautam *et al*., 2015). In the present study, we have characterized *Ca*. P. aurantifolia (16Sr II), another phytoplasma associated with the phyllody disease of gerbera in India.

Materials and Methods

Survey and collection of disease samples. Ten samples of gerbera plants showing typical phytoplasma-like symptoms were collected during a roving survey from farmers at Bangalore rural District, Karnataka State, India. Two leaf samples from symptomless plants adjacent to the symptomatic plants were also collected. Percent incidence of infected plants was assessed. The leaf samples collected from each plant were separately bagged and brought to the laboratory. Asymptomatic leaves from the gerbera grown in a polyhouse were used as a negative control for further studies.

DNA isolation and PCR amplification. Total genomic DNA was extracted from both symptomatic and asymptomatic gerbera leaf samples by using CTAB method (Doyle and Doyle, 1990). The total genomic DNA isolated from known phytoplasma-infected plants (sesame and brinjal) was used as positive control. The status of phytoplasma in gerbera samples was assessed through PCR using primer pair P1/P7 (Schneider *et al*., 1995) and R16F2n/R16R2 (Gundersen and Lee, 1996). In order to better characterize the gerbera phyllody phytoplasma strain, multigene sequence analysis was performed. The genes selected for amplification and characterization included the *rp* operon genes using rp(I)F1A/rp(I)R1A primer pair (Martini *et al*., 2007) and nearly complete phytoplasma translocation protein genes *Sec*Y and *Sec*A using primer pairs *Sec*YF1/*Sec*YR1 (Lee *et al*., 2010) and *Sec*Afor1/*Sec*Arev2 (Dickinson and Hodgetts, 2013), respectively. All PCR reactions were carried out as described by Venkataravanappa *et al*. (2017). The PCR-amplified products of 16SrRNA (1.8 kb), *Sec*Y (1.5 kb), *rp* (1.2 kb) and *Sec*A (1.3 kb) were cloned into pTZ57R/T cloning vector according to the protocol described by Venkataravanappa *et al*. (2019). The plasmids were isolated and sequenced using M13F/R primers at Meduaxin Genomics India Pvt. Ltd, Bangalore, India.

Comparative analyses of nucleotide sequences. The sequences of phytoplasma 16SrRNA, *Sec*Y, *rp* and *Sec*A genes were subjected to BLAST search for homologous DNA sequences available from the NCBI database. Our query sequences got most significant matches to 16SrRNA (Table S1a), *Sec*Y (Table S1b), *rp* gene (Table S1c) and *Sec*A gene (Table S1d) of existing members of the *Ca*. P. aurantifolia (16SrII) group, sequences of these along with other phytoplasmas were retrieved from the database for analysis. Then different conserved and non-conserved genes of phytoplasma isolate from present study were compared with respective nucleotide sequences of other phytoplasmas using SEAVIEW program (Galtier *et al*., 1996). Phylogenetic tree was constructed by using MEGA 7 software (Kumar *et al*., 2016) using the neighbor joining method with 1000 bootstrapped replications.

Virtual RFLP analysis and gel plotting. The virtual RFLP analysis of trimmed (F2nR2) sequences of 16SrRNA gene of Gerbera phyllody phytoplasma (GePP1) from the present study was done by using iPhyClassifier online tool (Zhao *et al*., 2009). The F2nR2 fragment of GePP1 was digested with 17 restriction enzymes used for phytoplasma 16SrRNA RFLP analysis (Wei *et al*., 2007, 2008). After *in-silico* restriction digestion, a virtual 3.0% agarose gel electrophoresis image was generated, which was used for subsequent RFLP pattern comparisons. Further, similarity coefficient of GePP1 and other phytoplasmas (16Sr II) was calculated using i*Phy*Classifier software (Wei *et al*., 2007).

Results and Discussion

The gerbera plants displaying typical phytoplasma-like symptoms such as modification of florets to green leaf-like structure, incomplete opening of flowers and stunted growth were noticed in different farmers' fields (Fig. 1a,b). The disease incidence varied from 15–20%. The phytoplasma infection in ten gerbera samples was confirmed through PCR using 16s RNA gene universal (P1/P7, 1.8 kb) and nested primers (R16F2n/R16R2, 1.2 kb). Similarly, amplification with non-conserved gene primers resulted in expected PCR amplicons of *Sec*Y (1.5 kb), Ribosomal protein (1.2 kb) and *SecA* (1.5 kb) genes. The PCR amplicons of ten samples corresponding to 16SrRNA (1.8 kb), *Sec*Y (1.5 kb), *rp* (1.2 kb) and *Sec*A (1.3 kb) were cloned and sequenced. Sequence alignment analysis indicated that sequences obtained from all ten samples were identical with each other in the respective genes. Therefore, one representative sequence of each gene 16SrRNA (accession number MG013980), *Sec*Y (MG013983) Ribosomal protein (*rp,* MH816956) and *Sec*A (MH816940) obtained from one sample (GePP1) was deposited in the GenBank. The obtained sequences matched most significantly to 16SrRNA (Table S1a), *secY* (Table S1b), *rp* (Table S1c) and *secA* (Table S1d) genes of phytoplasmas classified in the 16SrII group.

Comparison 16SrRNA gene sequence of GePP1 with corresponding region of sixty-one phytoplasmas belonging to different groups revealed that the GePP1 shared maximum nt identity of 97.1% with Eggplant big bud (JX483699) and less than 95% nt identity with several phytoplasmas belonging to *Ca*. P. aurantifolia group (16Sr II) (Table S1a). These results were well supported by the phylogenetic

Fig. 1

Gerbera plant showing phyllody and incomplete opening of flowers and stunted growth of the plant
The arrow indicates the petals of flowers turned green as compared to normal flower.

analysis showing 16SrRNA gene of GePP1 closely clustering with members of *Ca*. P. aurantifolia group (16SrII) infecting different crops (Fig. 2a). Virtual RFLP patterns obtained from the GePP1 after *in-silico* digestions using seventeen restriction endonucleases were compared with previously reported *Ca*. P. aurantifolia (16SrII) groups/subgroups. The analysis showed differences from other reported subgroups in restriction profiles with enzymes *Bfa*I, *Hha*1, *Sau*3AI and *Rsa*I; GePP1 phytoplasma showed the maximum nt identity (99.5%) with *Ca*. P. aurantifolia (Fig. 4). The phytoplasma under study was a new strain with similarity coefficient less than or equal to 0.97 with all existing representative strains of the 16SrII group (Wei *et al*., 2007, 2008) and may be considered as new subgroup or a variant in subgroup 16SrII-D. This was further supported by the restriction map of GePP1 obtained from pDRAW32 (AcaClone Software; http://www.acaclone.com) analysis showing significant differences in *Bfa*I, *Hha*1, *Sau*3AI and *Rsa*I restriction enzyme analysis from Eggplant big bud phytoplasmas (JX483699) and other 13 representatives of subgroups 16Sr II- A, B, C, D, F, G, H, I, J, K, L, S, T belonging to 16Sr II (data not shown). This is the first report of *Ca*. P. aurantifolia belonging to 16Sr II subgroup infecting gerbera in India.

Fig. 2

Phylogenetic tree based on sequences of 16S rRNA (a) and *secY* gene (b) of Gerbera phyllody phytoplasma with the phytoplasma strains (Tables S1a and S1b) using the neighbor-joining algorithm

Horizontal distances are proportional to sequence distances, vertical distances are arbitrary. The trees were rooted with *Acholeplasma laidlawii* (U14905) and *Leptospira interrogans* (MH376290). A bootstrap analysis with 1000 replicates was performed and the bootstrap percent values more than 50 are numbered along branches.

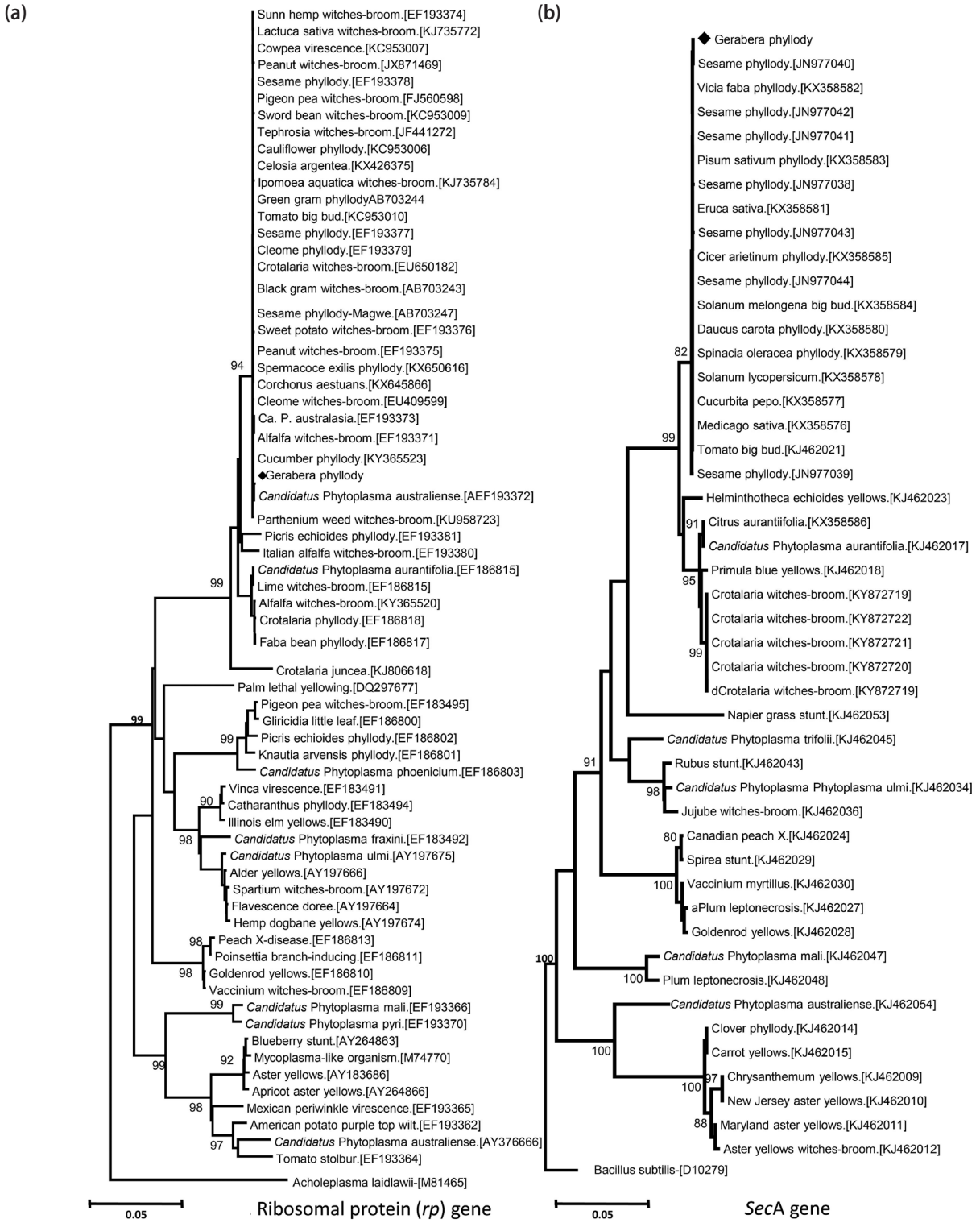

Fig. 3

Phylogenetic tree based on sequences of ribosomal protein (a) and *secA* (b) genes of Gerbera phyllody phytoplasma with other phytoplasma strains (Tables S1c and S1d) using neighbor-joining algorithm

Horizontal distances are proportional to sequence distances, vertical distances are arbitrary. The trees were rooted with *Acholeplasma laidlawii* (M81465) and *Bacillus subtilis* (D10279). A bootstrap analysis with 1,000 replicates was performed and only values above 50 shown.

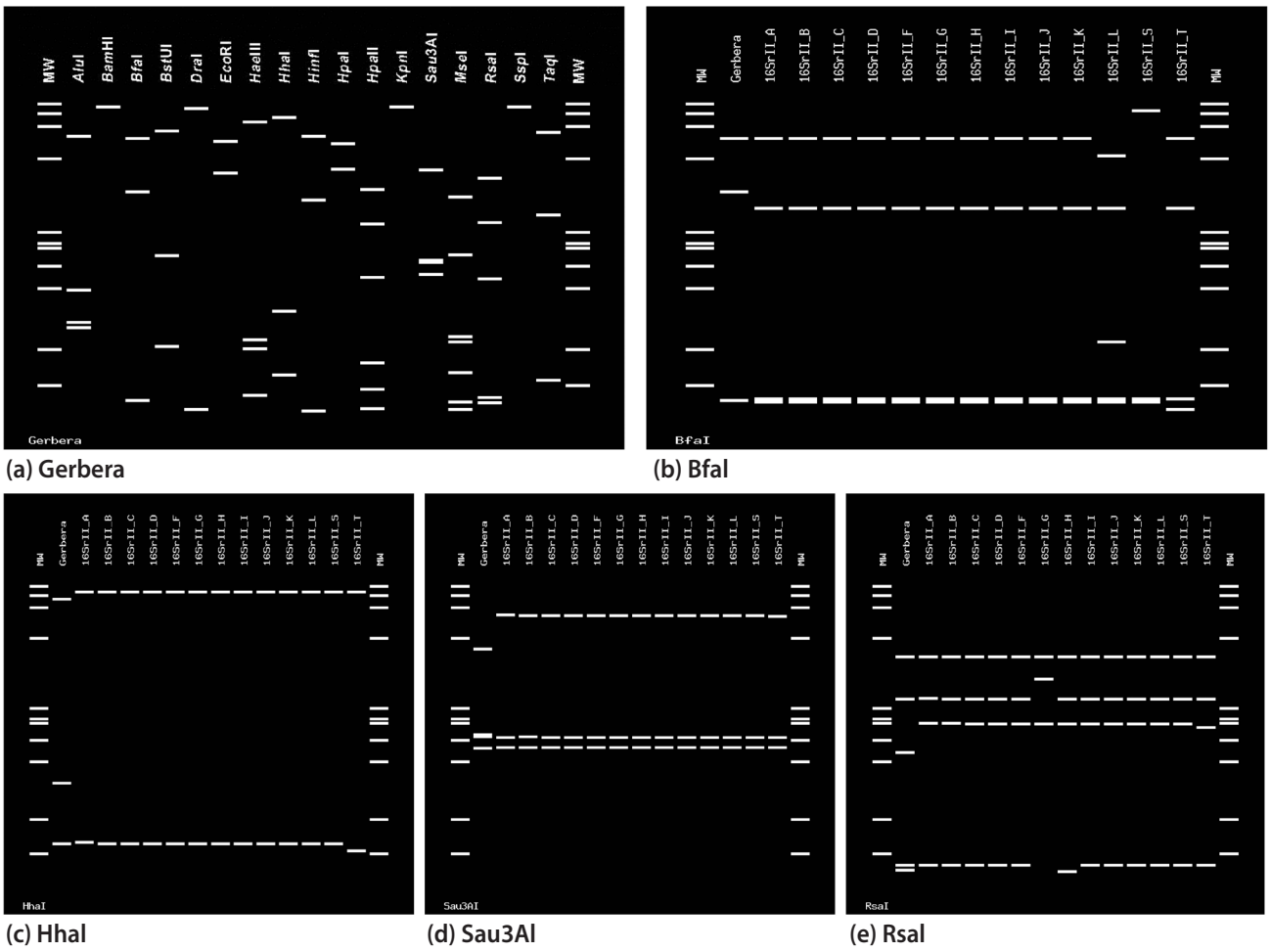

Fig. 4

Virtual RFLP patterns derived from *in silico* digestions, using *i*PhyClassifier for R16F2n/R16R2 fragment of 16S rRNA gene from Gerbera phyllody phytoplasma (strain GePP1)

The virtual RFLP patterns of GePP1 (*Bfa*I, *Hha*1, *Sau*3AI and *Rsa*I) distinguish the strain from those in a number of subgroups in group 16SrII. The restriction fragments were resolved through 3% virtual agarose gel. M: Molecular Ladder phiX174 DNA *Hae*III digest.

*Sec*Y gene of GePP1 was compared with the corresponding region of fifty-six phytoplasmas belonging to different groups (Lee *et al*., 2010). This comparison showed that GePP1 has maximum nt identity of 98.7 to 98.8% with Tomato big bud (KT970083, KT970081, KC953016, KT970084, KT970082, KT970080, KT970078) and other phytoplasmas belonging to the *Ca*. P. aurantifolia group (16Sr II) (Table S1b). This was supported by phylogenetic analysis results showing *Sec*Y gene of GePP1 closely clustering with the Tomato big bud phytoplasma strains from India (KT970078-84, KC953016, KC953016) and other phytoplasmas belonging to the *Ca*. P. aurantifolia group (16Sr II) (Fig. 2b).

The *rp* gene sequence of GePP1 comparison with the corresponding region of sixty-nine phytoplasmas from different groups showed highest nt identity of 99.2 to 99.6% with Alfalfa witches-broom (EF193371) and other phytoplasmas belonging to the *Ca*. P. aurantifolia group (16Sr II) (Table S1c). This result was also well supported by a phylogenetic analysis showing GePP closely clustering with the *Ca*. P. australiense (EF193372) and Tomato big bud (EF193373) phytoplasmas belonging to the *Ca*. P. aurantifolia group (16Sr II) (Fig. 3a).

Similarly, *Sec*A gene was compared with the corresponding region of thirty-eight different phytoplasmas sequences retrieved from the NCBI, GenBank. This comparison showed that GePP1 had maximum nt identity of 98.7 % with Sesame phyllody phytoplasmas from Thailand belonging to the *Ca*. P. aurantifolia group (16Sr II) (Table S1d). This result was also well supported by a phyloge-

netic analysis showing close clustering of GePP1 with the Sesame phyllody phytoplasmas (Fig. 3b).

The phytoplasma associated with phyllody disease of gerbera was identified on the basis of conserved 16SrRNA gene and less conserved genes *Sec*Y, Ribosomal protein, and *SecA* sequence and *in-silico* restriction analysis (Zhao *et al*., 2009). These analyses revealed that the phytoplasma causing phyllody in gerbera in Karnataka (India) is a member of *Ca*. P. aurantifolia and belongs to the 16Sr II group. Finer classification and description of the biology and ecology of phytoplasmas that are closely related but belong to distinct strains cannot be easily resolved by the highly conserved 16SrRNA gene sequence alone (Duduk and Bertaccini, 2011). Therefore, less conserved markers including *Sec*A, imp, tuf, ribosomal protein (*rp*), *Sec*Y, and SAP11 genes, have been previously utilized for finer classification of closely related phytoplasmas within or between the existing 16S group or subgroups (Marcone *et al*., 2000; Martini *et al*., 2007; Hodgetts *et al*., 2008; Kakizawa *et al*., 2009; Lee *et al*., 2010; Makarova *et al*., 2012). The sequence analysis of *Sec*Y, ribosomal protein (*rp*) and *SecA* gene in our study confirmed that the GePP1 phytoplasma has close evolutionary relationship with the *Ca* P. aurantifolia (Zreik *et al*., 1995; Hodgetts *et al*., 2008; Makarova *et al*., 2012; Valiunas *et al*., 2015).

This study represents the first evidence of association of phytoplasma related to *Ca*. P. aurantifolia belonging to 16SrII with gerbera from Karnataka, India.

The phytoplasma diseases of gerbera are becoming a major constraint for its production and are gaining international importance. More studies are required to determine the source of inoculum, vectors involved in transmission and the economic impact of *Ca*. P. aurantifolia in flower gardens, which will help finding strategies to contain this pathogen.

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