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Phylogenetic analysis of "rose witches'-broom" phytoplasma from cultivated *Rosa damascena* in India representing a new subgroup V-B1 in 16S rRNA gene group V

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ABSTRACT

Damask Rose (*Rosa damascena*; Family Rosaceae) is one of the most expensive essential oil bearing crops of many countries including India. A previously undescribed "rose witches'-broom" infestation was detected and exhibited symptoms of little leaf, apical proliferation and chlorosis during winter season in the experimental farms of CSIR-CIMAP, Lucknow (India). Samples from the healthy and infected plants were collected and indexed by PCR using the generic primer pairs P1/P6 and R16F2n/R16R2. The nested PCR product was cloned, sequenced and phytoplasma detected. The 16S rRNA gene sequence revealed that present phytoplasma showed maximum similarity of 97–98% with *Candidatus* Phytoplasma balanitae (HG937644), *Balanites triflora*' witches'-broom phytoplasma (BltWB) (AB689678) and Periwinkle yellows phytoplasma (EU375835), as well as other members of 16SrV group. Phylogenetic analysis of the 16S rRNA gene sequences of phytoplasma from Damask rose clustered with 16SrV phytoplasma group. However, computer-simulated RFLP analysis revealed unique profile of the phytoplasma sequence from rose with *Bam*HI, *Hpa*I and *Mse*I and distinguished it from Periwinkle phytoplasma, *Ca.* P. Balanitae, *Balanites triflora*' witches'-broom phytoplasma and all previously described '*Candidatus* Phytoplasma' of 16SrV groups. Further, the pattern similarity coefficient value was 0.55, lower than 0.85 with the representative phytoplasmas classified previously in 16SrV groups. Taking into consideration the unique plant host, RFLP profile and the restricted geographical occurrence in addition to the 16S rRNA gene sequence, the present phytoplasma is proposed to be rose witches'-broom phytoplasma representing a novel taxon 16SrV-B1. This is the first record of phytoplasma infection on Damask Rose from India.

1. Introduction

Phytoplasmas are pleomorphic, wall-less prokaryote and obligate plant pathogens that fall under the class Mollicutes (Seemüller et al., 1998; Bertaccini, 2007). These are small enough to pass through bacteriological filters and are the simplest self-replicating organisms. They normally inhabit the sieve tubes of phloem cells and more rarely, the parenchymal cells at low concentrations. The organism normally depicts an uneven distribution in monocots (Firrao et al., 2007) and transmitted by sap feeder insects belonging to the family Cicadellidae, Fulgoridae or Psyllidae (Weintraub and Beanland, 2006). The discovery of this new group of plant pathogens related to bacteria as mycoplasma-like organism or phytoplasma has prompted a new direction of studies to understand its morphology and etiology (Namba, 2011). These are now assigned to genus '*Candidatus* Phytoplasma'. Phytoplasmas are associated with the occurrence of hundreds of diseases in various economically important plant species including food, vegetable, and fruit crops; ornamental plants; trees (Bertaccini and Duduk, 2009) and medicinal and aromatic plants (Samad et al., 2006).

The attempts to isolate and cultivate phytoplasma in cell-free media have been futile. The measurable phenotypic characters remain largely inaccessible for phytoplasma taxonomy and classification (Lee et al., 1998a, 1998b; Hodgetts et al., 2007). Therefore, the genes with different degrees of nucleotide sequence conservation have been used to assess the genetic relatedness and phylogenetic relationships of diverse phytoplasmas. 16S rRNA gene sequences serve as the primary character for phytoplasma molecular taxonomy under the provisional status *Candidatus* for incompletely described prokaryotes (IRPCM, 2004).

Plants infected with phytoplasma exhibit symptoms including yellowing, witches'-broom, leaf curl, abnormal elongation of internodes,

Abbreviations: RWB, rose witches'-broom; RFLP, restriction fragment length polymorphism; CTAB, cetyltrimethylammonium bromide; TEM, transmission electron microscope; BLAST, basic local alignment search tool; PCR, polymerase chain reaction.

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floral virescence and distortion, shoot proliferation, plant sterility, phyllody, formation of bunchy fibrous secondary roots, reddening of leaves and stems, generalized yellowing, plant stunting and phloem necrosis (Ikten et al., 2014). Phytoplasma diseases cause significant yield losses in a large number of economically important crops worldwide (Bertaccini, 2007; McCoy et al., 1989; Kirkpatrick, 1992; Lee et al., 2000; Hogenhout et al., 2008).

The detection of a variety of phytoplasmas has now become easier by using polymerase chain reaction (PCR) for amplifying conserved regions such as 16S rRNA, ribosomal protein, *tuf* and 16S-23S spacer (Bertaccini and Duduk, 2009).On the basis of restriction fragment length polymorphism (RFLP) analysis of 16S rRNA, a genetically diverse group of phytoplasmas has been classified into groups and subgroups; each distinct group representing at least one putative phytoplasma species. Phylogenetic studies have suggested that phytoplasmas have descended from gram-positive bacteria ancestors and constitute a monophyletic clade in the class Mollicutes. Emerging information on phytoplasma diversity, their phylogenetic relationships, lineages, and taxonomy involving descriptions of "*Candidatus* Phytoplasma species" has emerged from the applications of molecular tools (Montano et al., 2001; White et al., 1998). The introduction of computer simulated RFLP analysis has also led to the identification and classification of diverse phytoplasmas (Wei et al., 2007, 2008). Till date, thirty-two 16S rRNA (16Sr) groups and more than 100 subgroups have been identified and cataloged (Martini and Lee, 2013).

Rosa damascena (Family, Rosaceae), commonly known as "Damask rose" is one of the important ornamental and aromatic plants used in a wide variety of food, nutritional, flavoring and medicinal products (Gudin, 2000). Damask rose which is native to Bulgaria, having strong aroma and fragrance (Widrlechner, 1981), is mainly cultivated as one of the most expensive essential oil bearing crop in countries like Iran, Egypt, France, China, Turkey, Bulgaria, Morocco and India (Demirözer et al., 2009). Approx 90% rose flowers are used for rose oil, 5–6% for rose concrete and 3–4% for rose water. (Charan and Gupta, 2013; Jabbarzadeh and Khosh-Khui, 2005). Beside its perfuming importance, several pharmacological properties including anti-HIV (Mahmood et al., 1996), antibacterial (Basim and Basim, 2003) antioxidant, antitussive, hypnotic, antidiabetic, and relaxant effect on tracheal chains have also been ascribed to rose plant (Boskabady et al., 2011).

So far, no phytoplasma infection has been reported on *R. damascena*, whereas several other species of genus *Rosa* were reported to inhabit phytoplasma (Kaminâska et al., 2001; Kaminâska et al., 2003; Kaminâska and Sliwa, 2004; Chaturvedi et al., 2009b; Gao et al., 2008). The present study constitutes the first record of phytoplasma infection in *R. damascena* cultivated at CSIR-CIMAP research farm during winters (2013). The infected plants depicted typical symptoms of disease like excessive shoot proliferation, reduction in leaf size and general stunting. Our study deals with the molecular characterization of the genome, phylogenetic analysis of the new phytoplasma associated with a witches'-broom disease of rose (*R. damascena*) using computer simulated RFLP i.e. *i*PhyClassifier web tool (Zhao et al., 2009) and hence we propose that the rose witches'-broom (RWB) phytoplasma be considered as a novel '*Ca.* Phytoplasma' taxon.

2. Materials and methods

2.1. Source of phytoplasmal DNA & electron microscopy

Twenty-one naturally occurring, diseased Damask rose plants were collected from 4 different locations with various stages of infection from the farms of CIMAP, Lucknow (India) during the month of January–February, 2013. These malformed plants exhibited typical symptoms including irregular edges, yellowing leading to narrow rolled and fragile leaves, stunted growth and dense clusters of highly proliferating apical shoot region. Symptomless rose plants from each of the 4 locations were also sampled as negative controls. Fresh infected and healthy leaf samples (2–3 mm) were excised and fixed in 3% gluteraldehyde and processed, and ultra thin sectioned were negatively stained with 2% uranyl acetate for examination under Jeol transmission electron microscope (TEM) at 80 kv as per the procedure of Tang and Faan (1987). Total DNA was extracted from each symptomatic and asymptomatic plant using the cetyltrimethylammonium bromide (CTAB) method as previously described (Khanuja et al., 1999). DNA preparations were stored at −20 °C until further use.

2.2. Polymerase chain reaction, sequence determination and phylogenetic analysis

PCR assay was performed for the detection of phytoplasma using universal primer pair P1/P6 (Deng and Hiruki, 1991). Nested-PCR assay amplifies the product of universal primer pair with a second set of primer pair R16F2n/R2 (Gundersen and Lee, 1996). Negative controls consisted of reaction mixtures devoid of templates. These reactions were carried out in a final volume of 25 μl, according to a methodology described previously (Lee et al., 1993). PCR products were analyzed by electrophoresis through 1% agarose gel.

The amplified nested PCR product was ligated into pGEMT Easy Vector System I (Promega) according to manufacturer's instructions and cloned in *Escherichia coli* DH5α. The cloned DNA fragments were sequenced with an automated sequencer (ABI Prism Perkin Elmer) as described by Sanz et al. (1999) at CIMAP, Lucknow. The nucleotide sequences obtained were aligned and subjected to BLASTn analysis (http://blast.ncbi.nlm.nih.gov/blast.cgi), while the phylogenetic analysis was performed by Clustal-X v2.1 (http://www.clustal.org/clustal2/) and MEGA v5 (http://www.megasoftware.net/) software. Sequences of the 16S rRNA gene belonging to phytoplasma of different subgroups from different hosts were used for phylogeny using neighbor-joining method. Bootstrapping was performed 1000 times and *Acholeplasma laidlawii* was included as an outgroup taxon to root the tree.

These nucleotide sequences were shortened to approximately 1.24 kbp region i.e., F2nR2 (the nucleotide sequence bounded by the small conserved motifs that corresponds to the annealing sites of universal 16S rRNA primer pair R16F2n/R16R2 for phytoplasma) for virtual RFLP analysis (http://plantpathology.ba.ars.usda.gov/virtualgel.html).

2.3. Computational virtual RFLP analysis

Computer-simulated RFLP analysis of 16S rRNA gene F2nR2 regions was performed using sequences of phytoplasma from damask rose and compared with *Candidatus* Phytoplasma balanitae (HG937644), *Balanites triflora*' witches'-broom phytoplasma (BltWB) (AB689678) and Periwinkle yellows phytoplasma (EU375835), available in the GenBank nucleotide database. *In silico* restriction analysis and virtual RFLP plotting were performed using pDRAW32 software (http://www.acaclone.com) and *i*PhyClassifier (http://www.ba.ars.usda.gov/data/mppl/) software (Zhao et al., 2009). Each sequence was digested in silico with 17 restriction enzymes plotted in a virtual 3.0% agarose gel. The virtual RFLP patterns were compared and a similarity coefficient (F) was calculated for each pair of phytoplasma strains according to the formula F52Nxy / (Nx + Ny), as previously described (Lee et al., 1998a, 1998b; Nei & Li, 1979). Putative phytoplasmas were routinely differentiated on the basis of 16S rRNA gene by means of RFLP analysis of PCR-amplified DNA sequences using a number of endonuclease restriction enzymes (Lee et al., 1998a, 1998b) as the RFLP pattern of each phytoplasma is conserved. The virtual RFLP patterns with the key enzymes that distinguish from previously recognized group/subgroup patterns were made in *i*PhyClassifier (http://www.ba.ars.usda.gov/data/mppl/). These virtual RFLP patterns were compared and similarity coefficient calculated by *i*PhyClassifier (http://www.ba.ars.usda.gov/data/mppl/) software (Wei et al., 2007).

3. Results

3.1. Symptomatology and detection of pathogen

Infected rose plants exhibited typical phytoplasma disease symptoms such as dwarfed and malformed narrow rolled and fragile leaves with irregular edges, leaf tip necrosis, excessive shoot proliferation, shortened petioles, fully/partly sterile flowers, and light yellowish coloration of leaves resembling those caused by phytoplasma infection (Fig. 1). Disease incidence was recorded about 18–22% on the basis of plant population in the fields. Typical phytoplasma-like (pleomorphic) bodies ranging in size from 450–900 nm were observed in the phloem cells of infected plants through transmission electron microscopy. The shape of these bodies varies as spherical, oval and tubular (Fig. 2). No other microorganism such as walled bacteria, fungus, virus or virus-induced structures, was noted. The healthy and/non-infected plants did not show these diagnostics features.

3.2. PCR, cloning and sequence determination

Total genomic DNA extracted from the leaves of symptomatic as well as asymptomatic plants, using CTAB method generated amplicons of 1.5 and 1.2 kb from the symptomatic plants but not from the healthy and water control reaction (Fig. 3). The nested PCR product of 1.25 kb was cloned into a pGEM-T cloning vector and sequence deposited in NCBI GenBank with Acc. no. KJ684064. The blast result showed high similarity of 98% with *Ca.* P. balanitae (HG937644) from India, Balanites triflora witches' broom BltWB (AB689678) from Myanmar and 97% with periwinkle yellow phytoplasma (EU375835) from China of 16SrV group phytoplasmas.

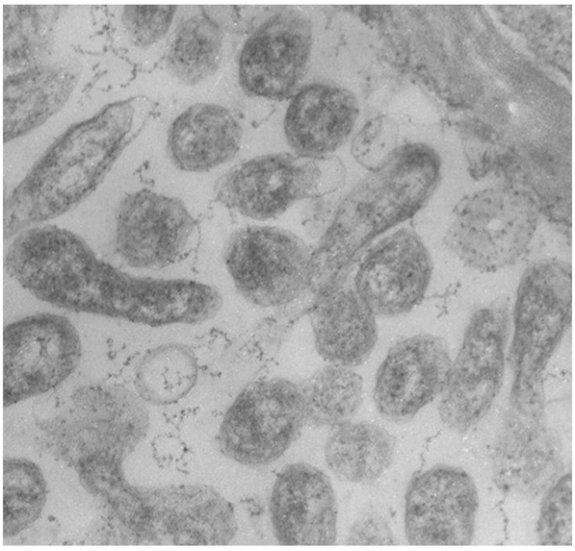

Fig. 2. Transmission electron micrograph showing pleomorphic bodies within a sieve tube of diseased *rose* plant.

3.3. Phylogenetic analysis

Phytoplasma sequence of 16S rRNA gene identified from *R. damascena* was compared with 18 sequences from different 16S rRNA phytoplasmal isolates of group '*Ca.* Phytoplasma' including *A. laidlawii* as an out-group. Phylogenetic tree was constructed with Mega 5 software using the neighboring-joining method with 1000 time bootstrapping. The phylogenetic analysis of rose phytoplasma 16S rRNA sequences revealed that the *R. damascena* phytoplasma

Fig. 1. Natural characteristic phytoplasma symptoms on Damask rose plants in the field.

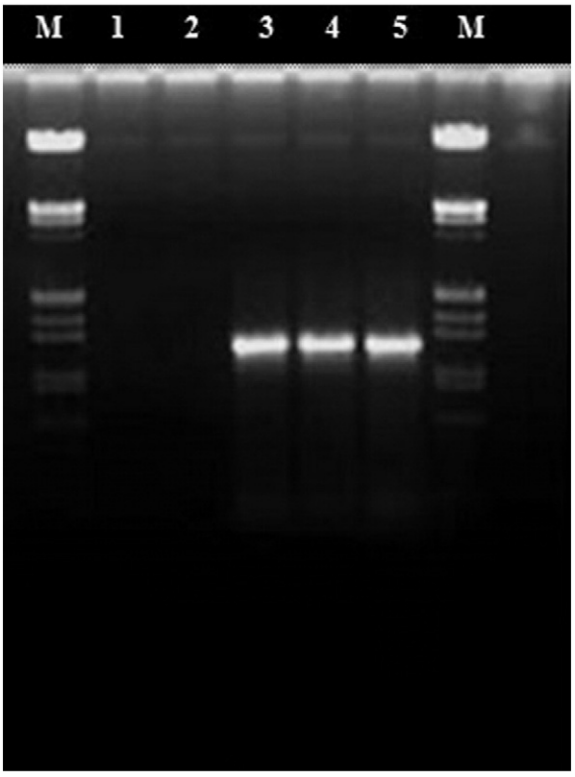

Fig. 3. Gel image showing the amplification (nested) of targeted fragment of DNA by PCR. M — λ DNA *Hind*III/*Eco*RI double digested marker, Well nos. 1, 2 — no amplification in healthy/negative control samples and Well nos. 3, 4, 5 — amplification of 1.2 kb fragment in infected samples.

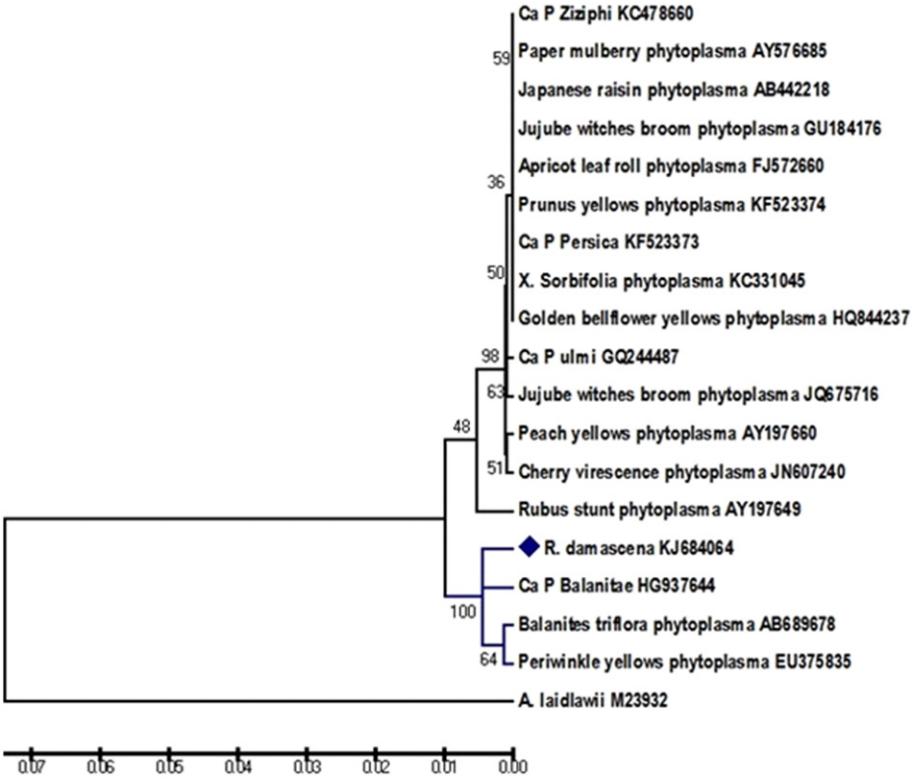

Fig. 4. Phylogenetic tree constructed with 16S rRNA gene sequences from previously described '*Candidatus* Phytoplasma' taxa. '*Ca.* P. balanitae' *Acholeplasma laidlawii* (GenBank accession no. M23932) was used as out group.

(KJ684064), *Candidatus* Phytoplasma balanitae (HG937644), *Balanites triflora*' witches'-broom phytoplasma (BltWB) (AB689678) and Periwinkle yellows phytoplasma (EU375835), shared a common ancestor of the same group of phytoplasmas-16SrV (Fig. 4). Though the results of sequence and phylogenetic analysis showed the maximum similarity with *Ca.* P. balanitae (HG937644) of 16SrV group, however, the results of *in silico* restriction digestion and gel plotting indicated delineation with this group and suggested to be a new subgroup lineage.

3.4. Virtual RFLP analysis

Although, maximum nucleotide matching (97–98%) revealed the close relationship of the present Damask rose phytoplasma with the *Balanites triflora*' witches'-broom phytoplasma (BltWB) (AB689678) and Periwinkle yellows phytoplasma (EU375835) and of 16SrV group of phytoplasmas. However, the virtual RFLP patterns of the 16S rDNA sequence of phytoplasma of *R. damascena* were clearly distinct from the closely related members of 16SrV group. The genetic restriction map of the phytoplasma sequence of *R. damascena* (KJ684064) showed significant differences with the close related *Ca.* P. balanitae (HG937644), and Periwinkle yellows phytoplasma (EU375835) along with other 6 representatives of subgroups (16SrV-A, B, C, D, E & G) of 16SrV in the pattern of restriction enzyme sites with pDRAW32 (AcaClone Software; http://www.acaclone.com (Fig. 5). The virtual RFLP profile of the 16S rRNA gene of Damask rose phytoplasma was similar with majority of the restriction enzyme. However, *Hpa*I, *MSe*I and *BamH*I have expressed distinct RFLP profile when comparing the present isolated phytoplasma with the closely related (on the basis of nucleotide) phytoplasmas (*Ca.* P. balanitae & Periwinkle yellows phytoplasma) of 16SrV group.

According to the phytoplasma classification system, they are classified into different groups on the basis of similarity coefficients of 16S rRNA gene RFLP patterns (Lee et al., 1998a; Gudin, 2000; Wei et al., 2007). These 16S rRNA gene groups are further differentiated into subgroups. A threshold similarity coefficient for the new subgroup delineation was calculated to be 0.85 (Wei et al., 2008; Nejat et al., 2013). Thus, a new subgroup is recognized if phytoplasmal strain has a 0.85 or lower similarity coefficient with those of all existing representative strains of the given group (Wei et al., 2008; Nejat et al., 2013). Since similarity coefficient value of *R. damascena* phytoplasma (KJ684064) has been found to be 0.55 using *i*PhyClassifier, a new subgroup could be recognized. Execution of the program generated the result as matrix of similarity coefficients determined for all strain pairs (Table 1). Virtual RFLP analysis involving 16S rRNA gene F2nR2 fragments of related phytoplasma strains using three restriction enzymes (i.e. *BamH*I, *Hpa*I and *Mse*I) was also performed and the results suggested a significant distinct pattern (Fig. 6). These findings revealed unique RFLP profile and novelty of the phytoplasma strain from Damask rose suggesting it to be a new member as of 16SrV group.

4. Discussion

The present studies highlighted the first record of a new phytoplasma from Damask rose for the Elm yellows phytoplasma group (16SrV) from India. Members belonging to the Elm Yellows (16SrV group) share high 16S rRNA gene sequence resemblance (Davis and Dally, 2001), but the group also consists of phytoplasmas with an important variety of biological niches limited to woody perennial hosts. '*Candidatus* P. Ulmi' in the 16SrV-A subgroup is responsible for yellows of elm species in North America and Europe (Lee et al., 2004) and '*Candidatus* P. Ziziphi' in the 16SrV-B subgroup is the causal agent of jujube witches'-broom and cherry lethal yellows in Asia (Jung et al., 2003; Lee et al., 2004). In Europe, other phytoplasmas in the 16SrV group mainly infect grapevines (Maixner, 1994), alder (Lederer and Seemüller, 1991; Maurer et al., 1993), blackberry (de Fluiter and van der Meer, 1953; Maürer and Seemüller, 1995), species of the genus *Spartium* (Marcone et al., 1996) and *Clematis vitalba* (Angelini et al., 2004).

Earlier, different phytoplasmas have been reported on several ornamental or wild species/cultivars of rose (Kaminâska et al., 2001;

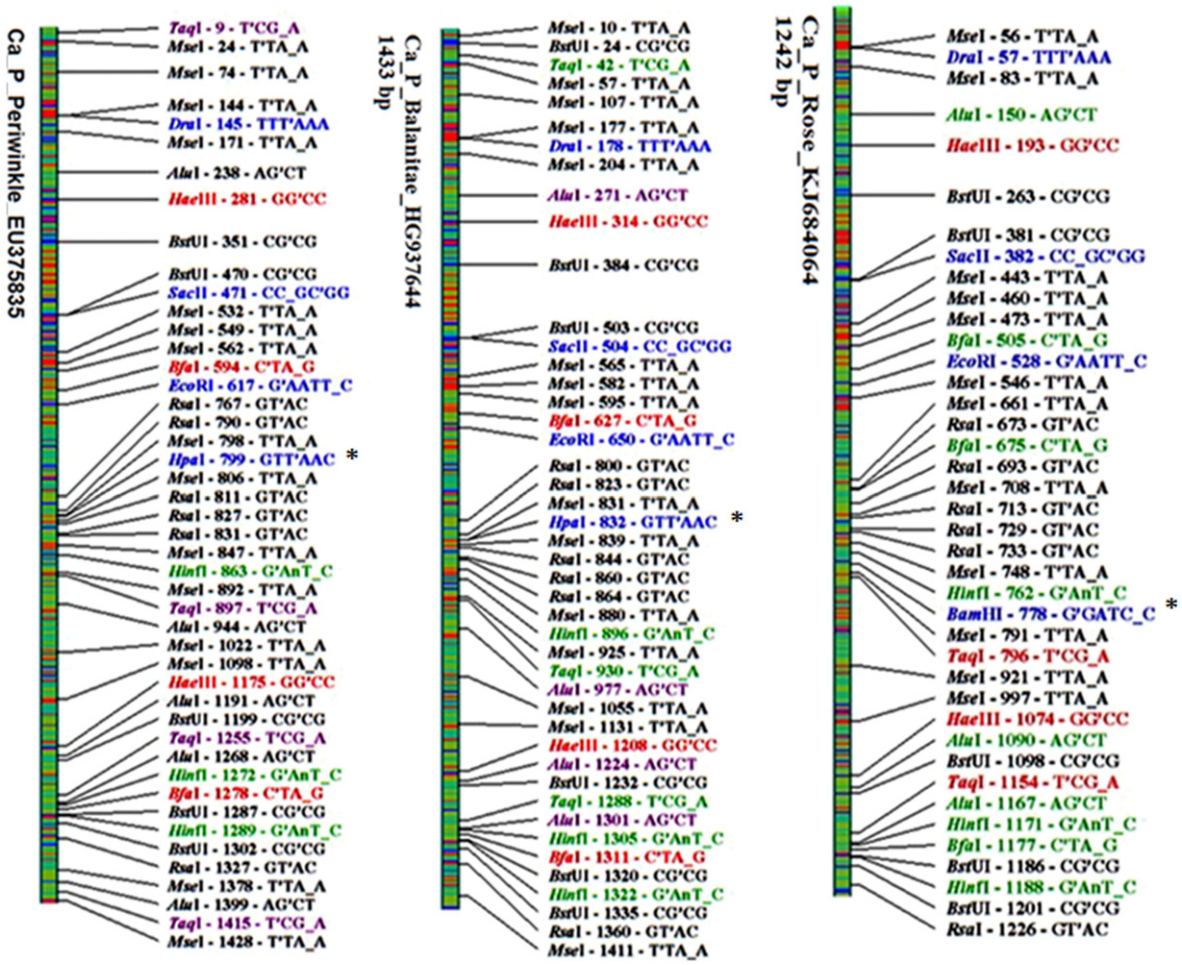

Fig. 5. Comparative analysis of virtual restriction sites in 16S rRNA gene. Sequences of 16S rRNA gene of phytoplasma from *R. damascena* (KJ684064), '*Ca.* P. balanitae' (HG937644) and Periwinkle yellows phytoplasma (EU375835). * highlights the important differences in the restriction sites.

Kaminâska et al., 2003; Kaminâska and Sliwa, 2004; Chaturvedi et al., 2009b; Gao et al., 2008) but detection of phytoplasma infection on *R. damascena* is another novelty of this work. The rose witches'-broom phytoplasma is proposed to be kept in a new subgroup (V-B1) of group 16SrV, as it possesses unique properties that are reflected in the results from enzymatic RFLP analyses, coefficients of similarity, and phylogenetic analysis based on 16S rRNA gene sequences. The analysis also suggested that RWB phytoplasma has a new and distinct lineage of 16SrV group. On BLAST, it showed the highest similarity with 97–98% *Candidatus* Phytoplasma balanitae (HG937644), *Balanites triflora*' witches'-broom phytoplasma (BltWB) (AB689678) and Periwinkle yellows phytoplasma (EU375835). According to the guidelines of the classification scheme (Lee et al., 1998b; Wei et al., 2008), a new group can be proposed if the collective RFLP pattern derived from the 16S rRNA gene F2nR2 fragment of a given phytoplasma strain has lower than 0.85 similarity coefficient values with the RFLP patterns of all previously recognized groups (Lee et al., 1998a; Hodgetts et al., 2007; Ikten et al., 2014; Nejat et al., 2013). The representative phytoplasma strains from *R. damascena* showed that similarity coefficient index of 0.55 as a new sub-group (V-B1) in 16S rRNA group V becomes justified.

Virtual RFLP analysis and generated data helped in the delineation of phytoplasma groups and in creation of strains as novel species. Present virtual RFLP pattern revealed that isolated phytoplasma from Damask rose (KJ684064) is different with RFLP pattern of representative strains of the same group 16SrV. The RFLP pattern with enzyme *Bam*HI, *Hpa*I and *Mse*I in particular provides the most distinguishing profile as

Table 1
Similarity coefficient derived from analysis of virtual RFLP analysis.

S. No.	Strain/Genbank accession	1	2	3	4	5	6	7	8	9	10	11	12
1	Ca_P_Balanitae_ HG937644	1.00											
2	*Balanites_triflora_* AB689678	0.97	1.00										
3	Periwinkle_yellows_EU375835	0.97	1.00	1.00									
4	*Pluchea_indica*_phytoplasma_KC778402	0.82	0.79	0.79	1.00								
5	*R._damascena*_KJ684064	0.59	0.58	0.58	0.59	1.00							
6	Apricot_leaf_roll_phytoplasma_FJ572660	0.95	0.91	0.91	0.78	0.55	1.00						
7	X._Sorbifolia_phytoplasma_KC331045	0.95	0.91	0.91	0.78	0.55	1.00	1.00					
8	Peach_yellows_phytoplasma_AY197660	0.95	0.91	0.91	0.78	0.55	1.00	1.00	1.00				
9	Prunus_yellows_phytoplasma_KF523374	0.95	0.91	0.91	0.78	0.55	1.00	1.00	1.00	1.00			
10	Ca_P_Ziziphi_KC478660	0.95	0.91	0.91	0.78	0.55	1.00	1.00	1.00	1.00	1.00		
11	Rubus_stunt_phytoplasma_AY197649	0.85	0.82	0.82	0.73	0.51	0.90	0.90	0.90	0.90	0.98	1.00	
12	Ca_P_Ulmi_GQ244487	0.95	0.91	0.91	0.78	0.55	1.00	1.00	1.00	1.00	0.88	0.90	1.00

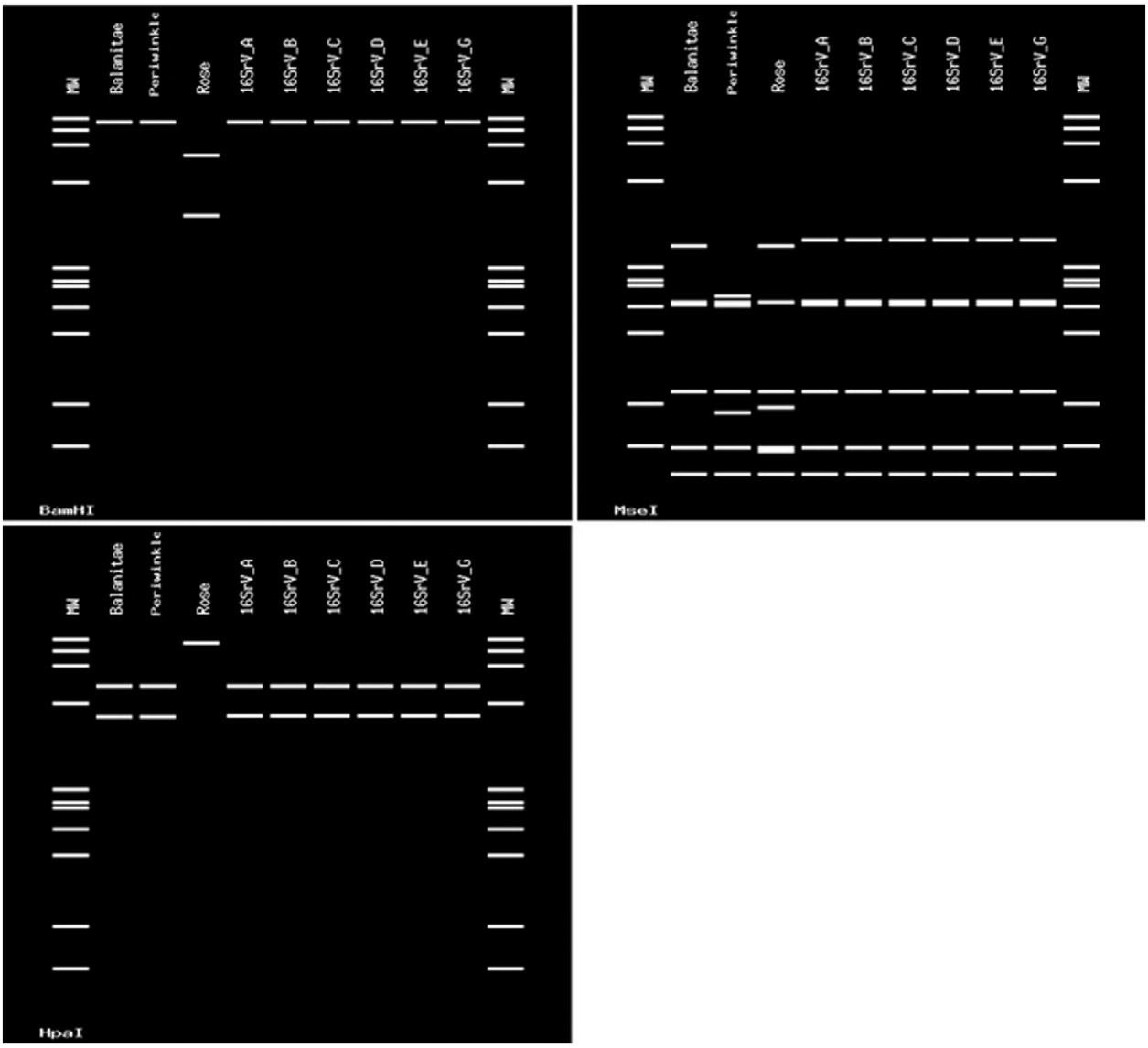

Fig. 6. Virtual RFLP presentation with key restriction enzymes. Virtual RFLP with *Mse*I, *Bam*HI and *Hpa*I distinguished rose witches'-broom (KJ684064) phytoplasma from the *Ca.* P balanite (HG937644), '*Pluchea indica* witches'-broom phytoplasma (KC778402), and six 16SrV group types.

compared to other members of 16SrV group. This phytoplasma represented a distinct lineage whose evolutionary history, host range, vectorship and other biological properties are yet to be explored. Nevertheless, the outcomes of the present study would extend the knowledge of 16SrV group phytoplasmas, their hosts and management strategies.

5. Conclusion

Damask Rose (*R. damascena*) is a commercially important valued crop for its essential oil. Recently, phytoplasma infection symptoms were observed in different commercial fields of CIMAP and other damask rose growing areas for the first time. We have characterized the associated pathogen via conventional and molecular techniques. The cloned fragment of the pathogen showed highest similarity of 97% to 98% with Periwinkle yellows phytoplasma (EU375835), *Candidatus* Phytoplasma balanitae (HG937644), and *Balanites triflora*' witches'-broom phytoplasma (BltWB) (AB689678). Sequence analysis of the isolated phytoplasma with software *i*phyclassifier and pDRAW 3.0 for virtual RFLP plotting, our current studies showed significant differences with reference to close related phytoplasma of Elm Yellows (16SrV) and revealed delineation from the group V-B. Prominent differences are recorded with enzymes *Bam*HI, *Hpa*I and *Mse*I and the similarity coefficient around 0.55 suggested that RWB is a new member of subgroup V-B1 in 16SrV group phytoplasma. This is the first record of a phytoplasm of 16SrV on Damask rose in India while earlier, it was reported from ecologically different places. These findings would be helpful in future studies for vector transmission and disease management.

Author contributions

STS did planning and execution of molecular experiments and data analysis. AKS helped in field survey, sample collection and computational analysis. AS helped in sample collection, wet lab experiments and collection of literature. AK helped in wet lab experiments (sequencing), analysis and manuscript preparation and AS did overall monitoring of the experiments and preparation of manuscript.

Conflict of interest

None.

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References

Angelini, E., Squizzato, F., Lucchetta, G., Borgo, M., 2004. Detection of a phytoplasma associated with grapevine "flavescence dorée" in *Clematis vitalba* L. Eur. J. Plant Pathol. 110, 193–201.

Basim, E., Basim, H., 2003. Antibacterial activity of *Rosa damascena* essential oil. Fitoterapia 74, 394–396.

Bertaccini, A., 2007. Phytoplasmas: diversity, taxonomy, and epidemiology. Front. Biosci. 12, 673–689.

Bertaccini, A., Duduk, B., 2009. Phytoplasma and phytoplasma diseases: a review of recent research. Phytopathol. Mediterr. 48, 355–378.

Boskabady, M.H., Shafei, M.N., Saberi, Z., Amini, S., 2011. Pharmacological effects of *Rosa damascena*. Iran J. Basic Med. Sci. 14, 295–307.

Charan, A.A., Gupta, P., 2013. Comparative analysis of antibacterial, antioxidant and photosynthetic activity of *Azadirachta indica*, *Rosa indica* and *Moringa oliefera* cultivars. Int. J. Curr. Chem. Res. 5, 556–561.

Chaturvedi, Y., Singh, M., Rao, G.P., Snehi, S.K., Raj, S.K., 2009b. First report of association of "*Candidatus* phytoplasma asteris" (16SrI group) with little leaf disease of rose (*Rosa alba*) in India. Plant Pathol. 58, 788.

Davis, R.E., Dally, E.L., 2001. Revised subgroup classification of group 16SrV phytoplasmas and placement of flavescence dorée-associated phytoplasmas in two distinct subgroups. Plant Dis. 85, 790–797.

de Fluiter, H.J., van der Meer, F.A., 1953. Rubus stunt, a leafhopper borne virus disease. Tijdschr Plantenziekten. 59, 195–197.

Demirözer, O., Kaydan, M.B., Karaca, I., Ben-Dov, Y., 2009. First records of armoured scale insects (Hemiptera: Coccoidea: Diaspididae) from the oil-rose, *Rosa damascena*, in Turkey. Hell. Plant Prot. J. 2 (1), 33–35.

Deng, S., Hiruki, C., 1991. Amplification of 16S rRNA genes from culturable and nonculturable mollicutes. J. Microbiol. Methods 14, 53–61.

Firrao, G., Garcia-Chapa, M., Marzachì, C., 2007. Phytoplasmas: genetics, diagnosis and relationships with the plant and insect host. Front. Biosci. 12, 1352–1375.

Gao, R., Zhang, G.M., Lan, Y.F., Zhu, T.S., 2008. Molecular characterisation of phytoplasma associated with rose witches'-broom in China. J. Phytopathol. 156, 93–98.

Gudin, S., 2000. Rose: genetics and breeding. Plant Breed. Rev. 17, 159–189.

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

Hodgetts, J., Ball, T., Boonham, N., Mumford, R., Dickinson, M., 2007. Use of terminol restriction fragment length polymorphism (T-RFLP) for identification of phytoplasmas in plants. Plant Pathol. 56, 357–365.

Hogenhout, A.S., Oshima, K., Ammar, D.E., Kakizawa, S., Kingdom, N.H., Namba, S., 2008. Phytoplasmas: bacteria that manipulate plants and insects. Mol. Plant Pathol. 9, 403–423.

Ikten, C., Catal, M., Yol, E., Ustun, R., Furat, S., Toker, C., Uzun, B., 2014. Molecular identification, characterization and transmission of phytoplasmas associated with sesame phyllody in Turkey. Eur. J. Plant Pathol. 139, 217–229.

IRPCM phytoplasma/spiroplasma working team – phytoplasma taxonomy group, 2004. 'Candidatus Phytoplasma', a taxon for the wall-less, non-helical prokaryotes that colonize plant phloem and insects. Int. J. Syst. Evol. Microbiol. 54, 1243–1255.

Jabbarzadeh, Z., Khosh-Khui, M., 2005. Factors affecting tissue culture of *Damask rose* (*Rosa damascena* Mill.). Sci. Hortic. 105, 475–482.

Jung, H.Y., Sawayanagi, T., Kakizawa, S., Nishigawa, H., Wei, W., Oshima, K., Miyata, S.I., Ugaki, M., Hibi, T., Namba, S., 2003. '*Candidatus* Phytoplasma ziziphi', a novel phytoplasma taxon associated with jujube witches'-broom disease. Int. J. Syst. Evol. Microbiol. 53, 1037–1041.

Kaminâska, M., Dziekanowska, D., Rudzinâska-Langwald, A., 2001. Detection of phytoplasma infection in rose, with degeneration symptoms. J. Phytopathol. 149, 3–10.

Kaminâska, M., Sliwa, H., 2004. First report of phytoplasma belonging to apple proliferation group in roses in Poland. Plant Dis. 88, 11.

Kaminâska, M., Sliwa, H., Malinowski, T., Skrzypczak, C., 2003. The association of aster yellows phytoplasma with rose dieback disease in Poland. J. Phytopathol. 151, 469–476.

Khanuja, S.P.S., Shasany, A.K., Darokar, M.P., Kumar, S., 1999. Rapid isolation of DNA from dry and fresh samples of plants producing large amounts of secondary metabolites and essential oils. Plant Mol. Biol. Report. 17, 1–7.

Kirkpatrick, B.C., 1992. Mycoplasma-like organisms: plant and invertebrate pathogens. In: Balows, H.G., Truper, M., Dworkin, W., Harder, K.H., Schleifer (Eds.), The Prokaryotes. Springer-Verlag, New York, pp. 4050–4067.

Lederer, W., Seemüller, E., 1991. Occurrence of mycoplasma-like organisms in diseased and non-symptomatic alder trees (*Alnus* spp.). Eur. J. For. Pathol. 21, 90–96.

Lee, I.M., Hammond, R.W., Davis, R.E., Gundersen, D.E., 1993. Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasmalike organisms. Phytopathology 83, 834–842.

Lee, I.M., Gundersen, D.E., Davis, R.E., Bartoszyk, I.M., 1998a. Revised classification scheme of phytoplasmas based on RFLP analysis of 16S rRNA and ribosomal protein gene sequences. Int. J. Syst. Bacteriol. 48, 1153–1169.

Lee, I.M., Gundersen-Rindal, D.E., Bertaccini, A., 1998b. Phytoplasma: ecology and genomic diversity. Phytopathology 88, 1359–1366.

Lee, I.M., Davis, R.E., Gundersen-Rindal, D.E., 2000. Phytoplasma: phytopathogenic mollicutes. Annu. Rev. Microbiol. 54, 221–255.

Lee, I.M., Martini, M., Marcone, C., Zhu, S.F., 2004. Classification of phytoplasma strains in the elm yellows group (16SrV) and proposal of '*Candidatus* Phytoplasma ulmi' for the phytoplasma associated with elm yellows. Int. J. Syst. Evol. Microbiol. 54, 337–347.

Mahmood, N., Piacente, S., Pizza, C., Burke, A., Khan, A.L., Hay, A.J., 1996. The anti-HIV activity and mechanisms of action of pure com-pounds isolated from *Rosa damascena*. Biochem. Biophys. Res. Commun. 229, 73–79.

Maixner, M., 1994. Transmission of German grapevine yellows (Vergilbungskrankheit) by the planthopper Hyalestes obsoletus (Auchenorrhyncha: Cixiidae). Vitis 33, 103–104.

Marcone, C., Ragozzino, A., Schneider, B., Lauer, U., Smart, C.D., Seemüller, E., 1996. Genetic characterization and classification of two phytoplasmas associated with spartium witches-broom disease. Plant Dis. 80, 365–371.

Martini, M., Lee, I.M., 2013. PCR and RFLP analyses based on the ribosomal protein operon. Methods Mol. Biol. 938, 173–188.

Maürer, R., Seemüller, E., 1995. Nature and genetic relatedness of the mycoplasma-like organism causing rubus stunt in Europe. Plant Pathol. 44, 244–249.

Maurer, R., Seemüller, E., Sinclair, W.A., 1993. Genetic relatedness of mycoplasmalike organisms affecting elm, alder, and ash in Europe and North America. Phytopathology 83, 971–976.

McCoy, R.E., Caudwell, A., Chang, C.J., Chen, T.A., Chiykowski, L.N., Cousin, M.T., Dale, D., Leeuw, G.T.N., Golino, D.A., Hackett, K.J., Kirkpatrick, B.C., Marwitz, R., Petzold, H., Sinha, R.C., Suguira, M., Whitcomb, R.F., Yang, I.L., Zhu, B.M., Seemüller, E., 1989. Plant diseases associated with mycoplasma-like organisms. In: Whitcomb, R.F., Tully, J.G. (Eds.), The Mycoplasmas. Academic Press, New York, pp. 546–640.

Montano, H.G., Davis, R.E., Dally, E.L., Hogenhout, S., Pimentel, J.P., Brioso, P.S.T., 2001. '*Candidatus* Phytoplasma brasiliense',a new phytoplasma taxon associatedwith hibiscus witches' broom disease. Int. J. Syst. Evol. Microbiol. 51, 1109–1118.

Namba, S., 2011. Phytoplasmas: a century of pioneering research. J. Gen. Plant Pathol. 77, 345–349.

Nejat, N., Vadamalai, G., Davis, R.E., Harrison, N.A., Sijam, K., Dickinson, M., Abdullah, S.N.A., Zhao, Y., 2013. '*Candidatus* Phytoplasma malaysianum', a novel taxon associated with virescence and phyllody of Madagascar periwinkle (*Catharanthus roseus*). Int. J. Syst. Evol. Microbiol. 63, 540–548.

Nei, M., Li, W.H., 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. 76, 5269–5273.

Samad, A., Shasany, A.K., Gupta, S., Ajayakumar, P.V., Darokar, M.P., Khanuja, S.P.S., 2006. First report of a 16SrVI group phytoplasma associated with witches'-broom disease on *Withania somnifera*. Plant Dis. 90, 248.

Sanz, A.I., Fraile, A., Gallego, J.M., Malpica, J.M., Garcia-Arenal, F., 1999. Genetic variability of natural populations of cotton leaf curl geminivirus, a single-stranded DNA virus. J. Mol. Evol. 49, 672–681.

Seemüller, E., Marcone, C., Lauer, U., Ragozzino, A., Goschl, M., 1998. Current status of molecular classification of the phytoplasmas. J. Plant Pathol. 80, 3–26.

Tang, W.F., Faan, H.C., 1987. An investigation on *Vinca rosea* as a host plant of citrus yellow shoot BLO. J. South China Agric. Univ. 8, 15–19.

Wei, W., Davis, R.E., Lee, I.M., Zhao, Y., 2007. Computer simulated RFLP analysis of 16S rRNA genes: identification of ten new phytoplasma groups. Int. J. Syst. Evol. Microbiol. 57, 1855–1867.

Wei, W., Lee, I.M., Davis, R.E., Suo, X., Zhao, Y., 2008. Automated RFLP pattern comparison and similarity coefficient calculation for rapid delineation of new and distinct phytoplasma 16Sr subgroup lineages. Int. J. Syst. Evol. Microbiol. 58, 2368–2377.

Weintraub, P.G., Beanland, L., 2006. Insect vectors of phytoplasmas. Annu. Rev. Entomol. 51, 91–111.

White, D.T., Blackall, L.L., Scott, P.T., Walsh, K.B., 1998. Phylogenetic positions of phytoplasmas associated with dieback, yellow crinkle and mosaic diseases of papaya, and their proposed inclusion in '*Candidatus* Phytoplasma australiense' and a new taxon,'*Candidatus* Phytoplasma australasia'. Int. J. Syst. Evol. Microbiol. 48, 941–951.

Widrlechner, M.P., 1981. History and utilization of *Rosa damascene*. Econ. Bot. 35, 42–58.

Zhao, Y., Wei, W., Lee, I.M., Shao, J., Suo, X.B., Davis, R.E., 2009. Construction of an interactive online phytoplasma classification tool, iPhyClassifier, and its application in analysis of the peach X-disease phytoplasma group (16SrIII). Int. J. Syst. Evol. Microbiol. 59, 2582–2593.