Molecular Characterization and Phylogeny of a Phytoplasma Associated with Phyllody Disease of toria (*Brassica rapa* L. subsp. *dichotoma* (Roxb.)) in India

M. Azadvar · V. K. Baranwal

Received: 1 November 2010 / Accepted: 12 January 2011 / Published online: 12 February 2011
© Indian Virological Society 2011

Abstract Samples from toria plants (*Brassica rapa* L. subsp. *dichotoma* (Roxb.)) exhibiting phyllody, virescence, witches broom, extensive malformation of floral parts, formation of bladder like siliquae and flower sterility were collected from four different locations in India. Sequencing and phylogenetic analysis of the 16S rRNA, a part of 23S rRNA, partial *sec A* genes, *rp* gene and 16S–23S intergenic spacer region indicated that the phytoplasmas associated with toria phyllody (TP) symptoms were identical and belonged to 16SrIX phytoplasma Pigeon pea witches’-broom (PPWB) group. The *iPhyClassifier* generated virtual RFLP pattern of 1.25 kb 16S rDNA sequences indicated that TP phytoplasma belongs to 16SrIX-C phytoplasma subgroup. Complete 23S rRNA gene of TP phytoplasma had 2,787 nucleotides and is the first sequence of 16SrIX phytoplasma group. Restriction digestion of 16S rDNA and 23S rDNA PCR products has also shown that TP phytoplasmas from all the four locations in India were identical. Toria is a previously unreported host for a phytoplasma in 16SrIX-C subgroup.

Keywords *B. rapa* · India · Phytoplasma · Sequencing · Toria · 16SrIX-C phytoplasma subgroup

Introduction

Toria (*Brassica rapa* L. subsp. *dichotoma* (Roxb.)), an oil-seed crop in India, has been affected by phyllody disease since 1958 [5, 6]. The associated phytoplasma was determined to be a member of 16SrIX Pigeon pea witches’-broom group phytoplasma on the basis of 16S rDNA sequencing [3].

Phytoplasmas have been classified according to 16S-rDNA phylogeny and RFLP profiles into 30 phylogenetic groups and 28 ‘*Candidatus* Phytoplasma’ species [31]. The phytoplasma strains within a species should share at least 97.5% sequence identity within the 16S rRNA gene (Firrao et al. 2005). Subsequently *in silico* virtual RFLP analysis has been used as a powerful tool for groups and subgroups phytoplasma identification [15, 29–31]. However, because of its highly conserved nature, the 16S rRNA gene may not be adequate for finer differentiation of closely related but distinct phytoplasmas strains. The additional markers such as *secY*, *tuf* and *rp* genes as well as 16S–23S intergenic spacer region genes sequencing have been used for subgroup differentiation of 16SrI phytoplasmas which were divided into 11 subgroups [7, 14, 17]. Similarly *tuf* gene and *rp* operon have been used to subdivide the members of 16SrXII phytoplasma group [24]. *SecY*, *map* and *uvrB–degV* genes were used to analyse strains in the 16SrV cluster [1]. As five subgroups in 16SrIX phytoplasma group have been identified [8, 19, 31], it was important to identify the phytoplasma subgroup associated with toria phyllody (TP) disease. Therefore, *in silico* virtual RFLP and phylogenetic analysis of 16S rRNA gene, actual RFLP of 16S rRNA and 23S rRNA genes, sequencing and phylogenetic analysis of ribosomal protein gene, 16S–23S rRNA intergenic spacer region, 23S rRNA and *secA* genes were attempted for characterization and phylogeny of a phytoplasma associated with TP disease using samples from four different locations in India.

M. Azadvar · V. K. Baranwal (✉)
Plant Virology Unit, Division of Plant Pathology, Indian
Agricultural Research Institute, New Delhi 110 012, India
e-mail: vbaranwal2001@yahoo.com

M. Azadvar
Plant Protection Department, Agricultural Research Center
of Jiroft and Kahnouj, 78615-115 Jiroft, Iran

Materials and Methods

Collection of Phyllody Samples

During 2009, plant samples of toria affected with phyllody were collected at the time of flowering from four major toria growing states of India viz., Delhi (Pusa campus, IARI, New Delhi), Uttar Pradesh (NRC for cropping system, Meerut), Punjab (Punjab Agricultural University, Ludhiana) and Rajasthan (Directorate of Rapeseed and Mustard, Bharatpur). Leaf midribs of symptomatic toria plants were used freshly or stored at -80°C for further study. Toria plants grown from the seeds in the glasshouse were used as healthy control.

Isolation of Total DNA and PCR Amplification

Total genomic DNA was isolated from 100 mg of leaf midrib tissues using DNeasy plant mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instruction. Five microliters of DNA was evaluated for phytoplasma DNA by a PCR assay employing universal phytoplasma primer pair P1/P7 [23]. For further confirmation, resulting amplification products were diluted to 1:30 with sterile distilled water. Two microliters were used for nested PCR with primer pair R16F2n/R16R2 [9]. The thermal conditions were as follows: denaturation at 94°C for 40 s (5 min for the first cycle), annealing at 55°C for 1 min and extension at 72°C for 2 min. The last cycle was extended for an additional 10 min at 72°C . Annealing temperature for nested PCR was 56°C . Subsequently a modified R16F2n forward primer (m(IX)R16F2n) was designed on the basis of sequence result of 16S rDNA product for better amplification of TP phytoplasma.

Amplification of 23S rRNA gene was performed using P23S5F3/A23S3R3 (5'-GTGGATGCCTTGGCACTAAGAGCC-3'/5'-ACTTACACACCTGGCCTATCAACC-3') primer pair as described by Guo et al. [10]. For amplification of *secA* gene, SecAfor1/SecArev3 (5'-GARATGAA AACTGGRGAAGG-3'/5'-GTTTTRGCAGTTCCTGTCAT NCC-3') phytoplasma universal primers were used [12]. PCR was also performed using 16SrIX group specific primer pair rp(IX)F2/rp(IX)R2 (5'-GCACAAGCTATTTTAA TGTTTACACC-3'/5'-CAAAGGGACTAAACCTAAAG-3') for amplification of a part of ribosomal protein (*rp*) gene. The thermal conditions were as follows: denaturation at 94°C for 1 min (5 min for the first cycle), annealing at 60°C for 2 min and extension at 72°C for 3.5 min. The last cycle was extended for an additional 10 min at 72°C [19].

All the PCR amplifications were carried out in a thermal cycler (Mycycler, Bio-Rad, USA) and the resulting PCR products were viewed in gel documentation unit (XR documentation system, Bio-Rad, USA).

Restriction Digestion of PCR Products

The PCR products of 16S rDNA (using R16F2n/R16R2 primer pair) and 23S rDNA (using P23S5F3/A23S3R3 primer pair) were purified by QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instruction. A total volume of 7 μl of PCR products were digested with restriction endonucleases *AluI*, *HaeIII*, *HhaI*, *HinfI*, *MseI* and *RsaI* (New England BioLabs, Waverley, MA, USA) at 37°C for 3 h. Resultant restriction fragments were visualized by electrophoresis through 2.8% agarose gel with Tris-EDTA as running buffer. DNA fragment profiles in gels were visualized and recorded in gel documentation unit (XR documentation system, Bio-Rad, USA).

Cloning of PCR Products and DNA Sequencing

PCR products obtained by use of different sets of primers were purified by QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany), as per manufacturer's instructions and cloned in pGEM-T Easy vector (Promega, USA) and transformed into *Escherichia coli* DH5- α cells (Invitrogen Life Technologies, Carlsbad, CA, USA). Recombinants were screened by blue and white screening method as per standard protocol [22]. Sequencing of 2–3 representative clones was performed with automated sequencing facility at Chromos Biotech (Bangalore, India). In addition purified PCR products were directly used for sequencing.

Phylogenetic Analysis

Sequences of PCR products and cloned DNA of PCR products were assembled using BioEdit software [11]. A database search of homologous sequences was performed by BLAST analysis at NCBI (<http://ncbi.nlm.nih.gov/BLAST>). 16S rRNA, 23S rRNA, *rp* and *secA* gene sequences were aligned with phytoplasma group/subgroup representatives available in GenBank using Clustal W [26]. Phylogenetic relationship of four isolates of TP phytoplasma with other group representative phytoplasma sequences available in GenBank were assessed based on sequences of 16S rRNA gene between primers R16F2n and R16R2, *secA* gene between primers SecAfor2 and SecArev3 [12], 23S rRNA gene between primers P7 and 23Srev [12], *rp* gene between primers rp(IX)F2 and rp(IX)R2 [19] and 16S–23S rRNA intergenic spacer region between primers P3 and P7 [12]. Sequence alignments were performed using CLUSTAL W [26]. The phylogenetic distance tree and molecular evolutionary analyses were performed with MEGA 4.0 software [25] using the maximum parsimony method with default values and 1,000 replications for bootstrap analysis. *Acholeplasma palmae* was used as out groups to root the trees.

In Silico Restriction Enzyme Digestion of 16S rDNA and Virtual Gel Plotting

1.25 kb of 16S rDNA sequences (R16F2n/R16R2 primer primed) derived from all the four TP phytoplasma strains from different locations of India were submitted to *iPhyClassifier* online tool (<http://www.plantpathology.ba.ars.usda.gov/cgi-bin/resource/iphyclassifier.cgi>) as described by Zhao et al. [30]. *In silico* restriction analysis and virtual gel plotting with restriction endonucleases *AluI*, *BamHI*, *BfaI*, *BstUI* (*ThaI*), *DraI*, *EcoRI*, *HaeIII*, *HhaI*, *HinfI*, *HpaI*, *HpaII*, *KpnI*, *Sau3AI* (*MboI*), *MseI*, *RsaI*, *SspI* and *TaqI* were obtained and compared with the virtual RFLP gel from 16SrIX phytoplasma subgroups by the same restriction enzymes [30].

Results

The major symptoms of phytoplasma diseases of toria consisted of flower sterility, virescence, phyllody, formation of hollow bladders and pod malformation (Fig. 1). An approximately 2.7 kb PCR product was obtained from four TP phytoplasma isolates using P23S5F3/A23S3R3 primer pair. No amplification obtained in healthy toria samples (Fig. 2).

Actual RFLP Analysis of 16S rDNA and 23S rDNA PCR Products

Nested PCR products of 16S rDNA using P1/P7 followed by R16F2n/R16R2 primers of TP phytoplasma isolates

from New Delhi, Meerut, Bharatpur and Ludhiana digested with *AluI*, *HaeIII*, *HhaI*, *HinfI*, *MseI* and *RsaI* endonuclease were completely identical (Fig. 3). Similarly PCR products of 23S rDNA of these isolates by P23SF3/A23SR3 primers produced the identical RFLP patterns. No differences in restriction fragment profiles were evident among all the four TP isolates indicating that they all contained the same phytoplasma (Fig. 4).

Sequence Analysis

16S–23S rDNA sequences of all the four TP phytoplasma isolates of India collected from New Delhi (GenBank Acc. GU11154), Meerut (HM559247), Bharatpur (HM559246) and Ludhiana (HM559245) were completely identical and had 1,845 nt. Comparison of 16S–23S rDNA sequences of four TP phytoplasma Indian isolates to other phytoplasma sequences revealed that the phytoplasma associated with TP had maximum identity of 99% to 16S rRNA gene sequence of Khafr (Iran) almond witches'-broom (DQ195209) and Pigeon pea witches'-broom (AF248957) and 98% to *Juniperus occidentalis* witches'-broom (GQ925918) phytoplasmas all belonging to 16SrIX phytoplasma group.

Comparison of 1.25 kb of F2n/R2 primed sequence of 16S rDNA of four TP phytoplasma isolates to other phytoplasma sequences reported in GenBank using the BLAST tool revealed that the phytoplasma associated with toria phyllody had maximum identity of 99% to *Knautia arvensis* phytoplasma, KAP strain (EF186823), Khafr (Iran) almond witches' broom (DQ195209) and some other phytoplasmas in 16SrIX group.

Fig. 1 Symptoms of phyllody disease on toria (*Brassica rapa* L. subsp. *dichotoma* (Roxb.)); **a** healthy plant, **b** flower sterility, phyllody, virescence, bladder-like pod and pod malformation on infected plant

Fig. 2 Agarose gel electrophoresis of PCR products amplified from phytoplasma infected toria with Primer pair P23S5F3/A23S3R3; H, healthy toria; TPN, toria phyllody isolate New Delhi; TPM, toria phyllody isolate Meerut; TPB, toria phyllody isolate Bharatpur; TPL, toria phyllody isolate Ludhiana; M, 1 kb DNA ladder

Comparison of 838 nucleotide sequences of TP phytoplasma obtained by SecAfor1/SecArev3 primers to other phytoplasma sequences revealed that the phytoplasma associated with TP had maximum identity of 80% to the

secA gene sequence of Jujube witches'-broom phytoplasma (GU471770) of 16SrV group. However, when 483 bp sequences of *secA* gene of TP phytoplasma (primed SecA-for2/SecArev3 primer pair) of all the four TP phytoplasma isolates (HM559249, HM559250, HM559251 and HM559252) were compared, they showed maximum identity of 93% to *secA* gene sequence of Pigeon pea witches'-broom phytoplasma (EU168746) of 16SrIX group.

Sequences of complete 23S rRNA gene of all the four TP phytoplasma isolates (HM559253, HM559254, HM559255) and HM559256) were also identical and had 2,787 nucleotides. Blast analysis of 23S rRNA gene sequence of TP phytoplasma isolates to other phytoplasma sequences revealed that the TP phytoplasma had maximum identity of 91% with Jujube witches'-broom phytoplasma (GU723425) and Loofah witches'-broom phytoplasma (AF086621) of 16SrV group.

Comparison of 808 bp ribosomal protein (*rp*) gene sequences of all the four TP phytoplasma isolates (HM559257, HM559258, HM559269 and HM559260) to other phytoplasma sequences revealed that the phytoplasma associated with TP had maximum identity of 99% to the *rp* gene sequence of *K. arvensis* phytoplasma, KAP strain (EF186801) which belongs to 16SrIX-C subgroup.

Fig. 3 Actual RFLP analyses of 1.25 kb of 16S rDNA nested-PCR products of TP phytoplasma (amplified using primer pair R16F2n/R16R2) digested with *Hinf*I, *Hae*III, *Rsa*I, *Alu*I, *Hha*I and *Mse*I restriction enzymes; TPN, toria phyllody strain New Delhi; TPM,

toria phyllody strain Meerut; TPB, toria phyllody strain Bharatpur; TPL, toria phyllody strain Ludhiana; M, phi X174/*Hae*III marker (1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72)

Fig. 4 Actual RFLP analyses of 2.7 kb of 23S rDNA PCR products of TP phytoplasma (amplified using primer pair P23S5F3/A23S3R3) digested with *HinfI*, *HaeIII*, *RsaI*, *AluI*, *HhaI* and *MseI* restriction enzymes; TPN, toria phyllody strain New Delhi; TPM, toria phyllody

strain Meerut; TPB, toria phyllody strain Bharatpur; TPL, toria phyllody strain Ludhiana; M, phi X174/*HaeIII* marker (1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72)

In Silico Analysis of 16S rDNA Sequences

Analysis of R16F2n/R16R2 primed 16S rDNA sequences of TP phytoplasma isolates using *iPhyClassifier* online tool [30] showed that all TP phytoplasma isolates collected from four different locations in India shared 99.12% identity with 16S rDNA sequence of the '*Ca. Phytoplasma phoenicium*' reference strain (AF515636) which indicates TP phytoplasma is a '*Ca. Phytoplasma phoenicium*'-related strain.

The virtual RFLP pattern derived from *in silico* analysis of R16F2n/R16R2 primed sequence of 16S rRNA of all the four Indian TP phytoplasma isolates using *iPhyClassifier* tool with 17 selected restriction enzymes showed that all four TP phytoplasma isolates had identical RFLP pattern with a similarity coefficient of 0.98 to the reference pattern of 16Sr group IX, subgroup C (Y16389) indicating phytoplasma associated with TP disease in India is a member of 16SrIX-C subgroup in the phytoplasma group classification scheme. Among the 17 restriction enzymes, *AluI*, *DraI*, *HaeIII*, *HhaI*, *HinfI*, *RsaI* and *TaqI* were differential restriction enzymes for subgroup identification in pigeon pea witches'-broom phytoplasma group and all of the 16SrIX group produced the same RFLP patterns when rest of the restriction enzymes were used (data not shown).

Phylogenetic Analysis

Phylogenetic analysis of 16S rDNA sequences showed that all the four TP phytoplasma isolates from India grouped together with 16SrIX-C phytoplasma subgroup, *K. arvensis* phytoplasma KAP strain (EF186823) and they in turn grouped with 16SrIX subgroups E (GQ925919), A (AF248957), D (AF515636) and B (AF455041) of 16SrIX group phytoplasma. All subgroups in the group 16SrIX clustered with *Cassia witches'*-broom (EF666054) of 16SrXXIX group (Fig. 5).

Discussion

Toria phyllody has been recorded in India since 1958 and causes reduced yield and oil content in TP infected plants by as much as 79 and 28% respectively [13]. The incidence of infection by phytoplasmas based on symptoms expressed in different accessions of toria was variable and reached 11% in toria field in New Delhi over a period of 2 years [2]. The major symptoms of phytoplasma diseases of toria consisted of virescence, phyllody and formation of hollow bladders but not stunting, leaf yellowing or purpling as reported for phytoplasma infected oilseed brassicas in

Fig. 5 Dendrogram constructed by the maximum parsimony method showing the phylogenetic relationships for 16S rDNA sequences (between primers R16F2n and R16R2) for TP phytoplasma isolates and representatives from other 16Sr groups. GenBank accession numbers are shown in parentheses. Bootstrap values greater than 70% (expressed as percentages of 1,000 replications) are shown

other countries [4, 16, 20] indicating that the phytoplasmas associated with toria phyllody might be distinct from phytoplasmas infecting brassicas in other countries. Comparison of 16S rRNA gene sequence of toria phyllody showed that the toria phyllody phytoplasma shared maximum identity of 99% to that of *K. arvensis* phyllody (Y18052) from Italy and Khafr (Iran) almond witches'-broom phytoplasma (DQ195209) from Iran. It showed 98% identity with Pigeon pea witches'-broom phytoplasma (AF248957) from the USA and 'Ca. Phytoplasma phoenicium' (AF515636) from Lebanon and Iran [27]. All these phytoplasmas belong to 16SrIX phytoplasma group. Phytoplasma associated with oilseed brassicas in Canada, Czech Republic, Italy and more recently in Greece on the other hand belonged to 16SrI group [4, 16, 20, 28]. Further study involving *in silico* analysis and virtual RFLP of 16S rDNA sequence including the application of *iPhyClassifier* indicated that all the four Indian isolates of TP phytoplasma were identical and belonged to 16SrIX-C phytoplasma subgroup and this was further supported by phylogenetic analyses of 16S rRNA, *rp* genes and 16S–23S rRNA intergenic region spacer sequences. Association of

16SrIX-C subgroup of phytoplasma is the first record with plants of *Brassicaceae* family. There are only two other phytoplasma in this sub group viz. phytoplasma associated with *K. arvensis* phyllody in Italy [18] and almond witches'-broom in Iran [21].

The 23S rRNA gene has not been sequenced completely for most of the phytoplasmas [10, 12]. Our study of complete 23S rRNA gene sequence from TP phytoplasma showed that it has maximum identity with Jujube witches'-broom phytoplasmas of 16SrV group. Whether rRNA gene, encoding 23S can be used for finer differentiation of phytoplasmas in a group or not will be known when more sequences of phytoplasma in a group become available. There was only one sequence of *secA* gene in 16SrIX group i.e. pigeon pea witches'-broom phytoplasma and TP phytoplasma had clustered with it but more sequences of *secA* gene would be required for its application for further sub grouping.

Acknowledgment We thank Dr. R.K. Jain, Head Division of Plant Pathology, for providing the facilities and Dr. D.K. Yadava, Division of Genetics, IARI, New Delhi for his kind help in sample collection.

References

- Arnaud G, Malembic-Maher S, Salar P, Bonnet P, Maixner M, Marcone C, Boudon-Padiou E, Foissac X. Multilocus sequence typing confirms the close genetic interrelatedness of three distinct flavescence dore'e phytoplasma strain clusters and group 16SrV phytoplasmas infecting grapevine and alder in Europe. *Appl Environ Microbiol.* 2007;73:4001–10.
- Azadvar M (2010). Biological and molecular characterization of phytoplasma associated with *Brassica rapa* in India. PhD Thesis, Indian Agricultural Research Institute, New Delhi.
- Azadvar M, Baranwal VK, Yadava DK. First report of a 16SrIX (Pigeon pea witches' broom) phytoplasma associated with toria (*Brassica rapa* cv. *toria*) phyllody disease in India. *New Dis Rep.* 2009;20:27.
- Bertaccini A, Vorácková Z, Vibio M, Fránová J, Navrátil M, Špak J, Nebesářová J. Comparison of phytoplasmas infecting winter oilseed rape in the Czech Republic with Italian Brassica phytoplasmas and their relationship to the aster yellows group. *Plant Pathol.* 1998;47:317–24.
- Bhowmik TP. Oilseed Brassicas constraints and their management. New Delhi: CBS Publishers and Distributors; 2003, p. 254.
- Bindra OS, Bakhetia DRC. A note on natural incidence of sesamum phyllody virus disease in *Brassica* spp. *Ludhiana J Res.* 1967;4:406–8.
- Botti S, Bertaccini A. Variability and functional role of chromosomal sequences in 16SrI-B subgroup phytoplasmas including aster yellows and related strains. *J Appl Microbiol.* 2003;94:103–10.
- Davis RE, Dally E, Zhao Y, Lee IM, Jomantiene R, Detweiler AJ, Putnam ML. First report of a new subgroup 16SrIX-E, '*Candidatus* Phytoplasma phoenicium'-related, phytoplasma associated with juniper witches' broom disease in Oregon. *New Dis Rep.* 2009;20:35.
- Firrao G, Gibb K, Streten C. Short taxonomic guide to the genus '*Candidatus* phytoplasma'. *J Plant Pathol.* 2005;87:249–263.
- Guo VH, Cheng ZM, Walla JA. Amplification of the 23S rRNA gene and its application in differentiation and detection of phytoplasmas. *Can J Plant Pathol.* 2000;22:380–6.
- Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. *Nucleic Acids Symp Ser.* 1999;41:95–8.
- Hodgetts J, Boonham N, Mumford R, Harrison N, Dickinson M. Phytoplasma phylogenetics based on analysis of *secA* and 23S rRNA gene sequences for improved resolution of candidate species of '*Candidatus* Phytoplasma'. *Int J Syst Evol Microbiol.* 2008;58:1826–37.
- Kaushik CD, Tripathi NN, Vir S. Effect of date of sowing on toria phyllody incidence and estimation of losses. *Haryana Agric Univ J Res.* 1978;8:28–30.
- Lee IM, Zhao Y, Bottner KD. SecY gene sequence analysis for finer differentiation of diverse strains in the aster yellows phytoplasma group. *Mol Cell Probes.* 2006;20:87–91.
- Lee IM, Zhao Y, Davis RE. Prospects to multiple gene-based systems for differentiation and classification of phytoplasmas. In: Weintraub PG, Jones P, editors. *Phytoplasmas genomes, plant hosts and vectors*. Wallingford: CAB International; 2010. p. 51–63.
- Maliogka VI, Tsialtas JT, Papantoniou A, Efthimiou K, Katis NI. First report of a phytoplasma associated with an oilseed rape disease in Greece. *Plant Pathol.* 2009;58:792.
- Marcone C, Lee IM, Davis RE, Ragozzino A, Seemüller E. Classification of aster yellows-group phytoplasmas based on combined analyses of rRNA and *tuf* gene sequences. *Int J Syst Evol Microbiol.* 2000;50:1703–13.
- Marcone C, Ragozzino C, Camele I, Rana GL, Seemüller E. Updating and extending genetic characterization and classification of phytoplasmas from wild and cultivated plants in southern Italy. *J Plant Pathol.* 2001;83:133–8.
- Martini M, Lee IM, Bottner KD, Zhao Y, Botti S, Bertaccini A, Harrison NA, Carraro L, Marcone C, Khan AJ, Osler R. Ribosomal protein gene based phylogeny for finer differentiation and classification of phytoplasmas. *Int J Syst Evol Microbiol.* 2007;57:2037–51.
- Olivier CY, Séguin-Swartz G, Hegedus D. First report of "*Candidatus* Phytoplasma asteris" related strains in *Brassica rapa* in Saskatchewan, Canada. *Plant Dis.* 2006;90:832.
- Salehi M, Izadpanah K, Heydarnejad J. Characterization of a new almond witches'-broom phytoplasma in Iran. *J Phytopathol.* 2006;154:386–91.
- Sambrook J, Russell DW. *Molecular cloning: a laboratory manual*. Cold Spring Harbor: Cold Spring Harbor Laboratory; 2001.
- Schneider B, Seemüller E, Smart CD, Kirkpatrick BC. Phylogenetic classification of plant pathogenic mycoplasma like organisms or phytoplasmas. In: Razin R, Tully JG, editors. *Molecular and diagnostic procedures in mycoplasmaology*, vol. I. San Diego: Academic Press; 1995. p. 369–80.
- Streten C, Gibb KS. Genetic variation in '*Candidatus* Phytoplasma australiense'. *Plant Pathol.* 2005;54:8–14.
- Tamura K, Dudley J, Nei M, Kumar S. MEGA 4.0: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol.* 2007;24:1596–9.
- Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties, and weight matrix choice. *Nucleic Acids Res.* 1994;22:4673–80.
- Verdin E, Salar P, Danet JL, Choueiri E, Jreijiri F, El Zammam S, Gélle B, Bové JM, Garnier M. '*Candidatus* Phytoplasma phoenicium' sp. nov., a novel phytoplasma associated with an emerging lethal disease of almond trees in Lebanon and Iran. *Int J Syst Evol Microbiol.* 2003;53:833–8.
- Wang K, Hiruki C. Molecular characterization and classification of phytoplasmas associated with canola yellows and a new phytoplasma strain associated with dandelions. *Plant Dis.* 2001;85:76–9.
- Wei W, Davis RE, Lee IM, Zhao Y. Computer-simulated RFLP analysis of 16S rRNA genes: identification of ten new phytoplasma groups. *Int J Syst Evol Microbiol.* 2007;57:1855–67.
- Zhao Y, Wei W, Lee IM, Shao J, Suo X, Davis RE. Construction of an interactive online phytoplasma classification tool, iPhy-Classifier, and its application in analysis of the peach X-disease phytoplasma group (16SrIII). *Int J Syst Evol Microbiol.* 2009;59:2582–93.
- Zhao Y, Wei W, Davis RE, Lee IM. Recent advances in 16S rRNA gene-based phytoplasma differentiation, classification and taxonomy. In: Weintraub PG, Jones P, editors. *Phytoplasmas genomes, plant hosts and vectors*. Wallingford: CAB International; 2010. p. 64–92.