Detection and identification of a novel subgroup 16SrII-V phytoplasma associated with *Praxelis clematidea* phyllody disease

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Abstract

*Praxelis clematidea* is a very vigorous non-native weed in tropical and subtropical regions of China. *P. clematidea* plants showing symptoms of phyllody disease were found in an orchard located in Hainan province, PR China. The presence of phytoplasmas was confirmed by PCR of 16S rRNA gene using phytoplasma universal primers R16mF2/R16mR1 followed by R16F2n/R16R2. According to 16S rRNA gene sequence similarity, the *P. clematidea* phyllody (PCP) phytoplasma is a *Candidatus Phytoplasma australasiae*-related strain (99.5% similarity). The virtual RFLP pattern analyses of 16S rRNA gene sequences indicated that the PCP is a new subgroup within 16Sr group II. The most similar RFLP pattern is the reference pattern of 16Sr group II, subgroup M, with a similarity coefficient of 0.94. These results were confirmed by phylogenetic analyses of the 16S rRNA gene. These findings suggest that *P. clematidea* phyllody disease is caused by a new phytoplasma considered to be a novel subgroup, 16SrII-V.

*Praxelis clematidea* (Griseb.) R. M. King and H. Rob. originated in South America and has become an invasive alien plant in China [1]. This weed has distinctive bluish purple flowers in small clusters and a very strong odour like cat urine when crushed. *P. clematidea* is similar in appearance to *Ageratum* but *P. clematidea* has stronger toothed leaves, shorter petioles and larger flower heads. Now, *P. clematidea* occurs as a common weed that grows vigorously in orchards, roadside verges, woods, fields and scrubland in Hainan province, PR China.

Phytoplasmas are established as an important group of plant pathogens. Based on 16S rRNA gene sequence, biological/phytopathological characteristics and RFLP patterns, 46 *Candidatus Phytoplasma* species, 34 16Sr groups and more than 100 subgroups have been described and identified in *Candidatus Phytoplasma* [2–4]. The peanut witches’ broom phytoplasma group (16SrII) is one of the most diverse phytoplasma groups, and 21 16SrII subgroups have been formally described already [5]. The symptoms of plants infected by phytoplasmas usually are yellowing, witches’ broom, phyllody, sterility of flowers, generalized stunting and phloem necrosis [6, 7]. A previous research study reported that phytoplasma pathogens are associated with *P. clematidea* witches’ broom disease [8]. In the present study, we obtained the 16S rRNA gene sequences of the phytoplasma associated with *P. clematidea* phyllody disease and classified this phytoplasma strain.

In 2016, we conducted disease surveys mainly on orchards in Chengmai county, Hainan province, PR China. We observed a suspected phytoplasma disease on *P. clematidea*. Diseased plants displayed phyllody and witches’ broom (Fig. S1, available in available in the online version of this article). Leaf samples were randomly collected from six symptomatic *P. clematidea* plants displaying typical phyllody symptoms. Similar samples were also collected from healthy plants grown in another orchard for use as negative controls.

Total nucleic acid was extracted from both infected and healthy *P. clematidea* plants using a phytoplasma enrichment and cetyltrimethylammonium bromide (CTAB) extraction procedure [9]. A nested PCR was used to detect phytoplasmas using the universal primers R16mF2/R16mR1 followed by R16F2n/R16R2 [10], which amplified a phytoplasma 16S rRNA gene segment (approximately 1.2 kbp). For both
Table 1. Similarity coefficients (F) derived from the *P. clematidea* phyllody phytoplasma-CM01 (16SrII-V), identified in this study, and reference phytoplasmas belonging to group 16Sr II

| Strain                        | GenBank no. | Classification | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  | 13  | 14  | 15  | 16  | 17  | 18  | 19  | 20  | 21  | 22  |
|-------------------------------|-------------|----------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 1 Peanut WB phytoplasma       | L33765      | 16SrII-A       | 1.00|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 2 *Ca. Phytoplasma aurantifolia* | U15442     | 16SrII-B       | 0.88| 1.00|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 3 Faba bean phyllody phytoplasma | X83432     | 16SrII-C       | 0.94| 0.94| 1.00|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 4 *Ca. Phytoplasma australasiea* | Y10097     | 16SrII-D       | 0.95| 0.91| 0.97| 1.00|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 5 *Pistis stichosis* phyllody phytoplasma | Y16393     | 16SrII-E       | 0.90| 0.85| 0.91| 0.94| 1.00|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 6 Cactus WB phytoplasma-YN11  | EU099356    | 16SrII-F       | 0.91| 0.93| 0.97| 0.94| 0.88| 1.00|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 7 Cactus WB phytoplasma-YN23  | EU099368    | 16SrII-G       | 0.91| 0.91| 0.97| 0.94| 0.88| 0.94| 1.00|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 8 Cactus WB phytoplasma-YN24  | EU099369    | 16SrII-H       | 0.90| 0.92| 0.96| 0.93| 0.87| 0.93| 0.95| 1.00|     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 9 Cactus WB phytoplasma-YN06  | EU099351    | 16SrII-I       | 0.90| 0.89| 0.95| 0.92| 0.87| 0.92| 0.92| 0.91| 1.00|     |     |     |     |     |     |     |     |     |     |     |     |     |
| 10 Cactus WB phytoplasma-YN07 | EU099352    | 16SrII-J       | 0.88| 0.88| 0.94| 0.91| 0.85| 0.91| 0.91| 0.90| 0.89| 1.00|     |     |     |     |     |     |     |     |     |     |     |     |
| 11 Cactus WB phytoplasma-YN28 | EU099372    | 16SrII-K       | 0.88| 0.88| 0.94| 0.91| 0.85| 0.91| 0.91| 0.90| 0.89| 0.88| 1.00|     |     |     |     |     |     |     |     |     |     |     |
| 12 Cactus WB phytoplasma-YN01 | EU099346    | 16SrII-L       | 0.87| 0.87| 0.92| 0.90| 0.86| 0.89| 0.89| 0.88| 0.87| 0.87| 1.00|     |     |     |     |     |     |     |     |     |     |     |
| 13 *Tephrosia purpurea* WB phytoplasma | HG792252   | 16SrII-M       | 0.81| 0.86| 0.92| 0.89| 0.85| 0.89| 0.89| 0.88| 0.87| 0.86| 0.86| 1.00|     |     |     |     |     |     |     |     |     |     |
| 14 Bunchy top symptom phytoplasma-II-LT | JF781309   | 16SrII-N       | 0.92| 0.92| 0.98| 0.95| 0.89| 0.95| 0.95| 0.94| 0.93| 0.92| 0.90| 0.91| 1.00|     |     |     |     |     |     |     |     |
| 15 *Tabebrada pentaphylla* phytoplasma | EF647744   | 16SrII-O       | 0.91| 0.91| 0.97| 0.94| 0.88| 0.94| 0.94| 0.93| 0.92| 0.91| 0.90| 0.89| 0.95| 1.00|     |     |     |     |     |     |     |
| 16 Cuban papaya phytoplasma   | DQ286948    | 16SrII-P       | 0.94| 0.85| 0.90| 0.90| 0.84| 0.87| 0.87| 0.86| 0.85| 0.83| 0.83| 0.88| 0.88| 1.00|     |     |     |     |     |     |     |
| 17 Papaya bunchy top phytoplasma-BTSphav02-IIA | JF781310   | 16SrII-Q       | 0.97| 0.86| 0.91| 0.92| 0.87| 0.88| 0.88| 0.86| 0.86| 0.84| 0.84| 0.90| 0.89| 0.92| 1.00|     |     |     |     |     |     |
| 18 *Ethnosipus sp. yellow patch phytoplasma* | DQ535900   | 16SrII-R       | 0.89| 0.88| 0.94| 0.91| 0.86| 0.91| 0.91| 0.90| 0.88| 0.88| 0.87| 0.94| 0.92| 0.93| 0.85| 0.86| 1.00|     |     |     |
| 19 *Amananthus hypochondriacus* phytoplasma-52A | FJ357164   | 16SrII-S       | 0.91| 0.91| 0.97| 0.94| 0.88| 0.94| 0.94| 0.93| 0.92| 0.91| 0.90| 0.97| 0.95| 0.94| 0.88| 0.89| 0.91| 1.00|     |     |
| 20 *Tomatillo WB phytoplasma*  | EU125185    | 16SrII-T       | 0.91| 0.91| 0.97| 0.94| 0.88| 0.94| 0.94| 0.93| 0.92| 0.91| 0.90| 0.89| 0.95| 0.94| 0.88| 0.89| 0.91| 0.94| 1.00|     |
| 21 *Papaya little leaf* phytoplasma-1201 | KP072502   | 16SrII-U       | 0.97| 0.85| 0.91| 0.92| 0.88| 0.88| 0.88| 0.87| 0.85| 0.85| 0.86| 0.85| 0.89| 0.88| 0.91| 0.94| 0.86| 0.88| 0.88| 1.00|
| 22 *P. clematidea* phyllody-CM01* | KY568717    | 16SrII-V       | 0.92| 0.81| 0.87| 0.88| 0.84| 0.83| 0.83| 0.82| 0.81| 0.79| 0.79| 0.85| 0.89| 0.90| 0.88| 0.88| 0.91| 0.90| 0.91| 1.00|

*The new subgroup (16SrII-V) is shown in bold type.
primer pairs, PCR was performed in 35 cycles of 1 min at 94 °C (4 min at 94 °C for the initial denaturation), 1 min at 50 °C, and 1 min 30 s at 72 °C, and a final extension cycle of 10 min at 72 °C. The PCR mixtures contained 1 U Taq DNA polymerase (TakaRa), 0.2 mM of each dNTP, 0.4 µM of each primer, and 1 µl nucleic acid preparation. One microlitre of diluted (1 : 30) PCR products from the first amplification was used as the template in the nested PCR. Healthy plant DNA sample and sterile water were used as the negative control and the blank control respectively.

Three PCR products from diseased plants were cloned and sequenced. For the cloning of 16S rRNA genes, the target DNA fragments were ligated into the plasmid vector pMD18-T (TakaRa) and the recombinant plasmid was used to transform *Escherichia coli* DH5α. The inserts (three inserts of each PCR product, nine inserts total) were sequenced with an automated DNA sequencer (ABI Prism model 3730XL; Applied Biosystems) using universal primer M13-47/RV-M (Sangon). Nucleotide sequence data revealed that there were two 16S rRNA genes that showed heterogeneity in *P. clematidea* phyllody phytoplasma. One of the obtained sequences (1245 nt), designated PCP-CM01 (*P. clematidea* phyllody-Chengmai 01), as representative strain, was deposited in GenBank (NCBI). This sequence was subjected to similarity analysis and computer-simulated RFLP analysis using the *i*PhyClassifier [11].

For phylogenetic analysis, 16S rRNA gene sequences from reference strains of the phytoplasma 16Sr group and from representative strains of 16SrII subgroups were retrieved from the NCBI database. Phylogenetic analysis was carried out using the minimum evolution approach of the MEGA5 software [12] as described by Tamura et al. [12].

Using nested PCRs, fragments of the expected size (1.2 kbp) were obtained from DNA samples of diseased plants. No amplification occurred with either primer pair when using samples collected from healthy *P. clematidea* plants and the

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**Fig. 1.** Virtual RFLP patterns of the 16S rRNA gene R16F2n/R16R2 fragments of *P. clematidea* phyllody phytoplasma. (a) Virtual RFLP patterns of 17 restriction endonuclease enzymes, (b) Msel, (c) AluI, (d) Bsal, (e) HaelII (f) HinfI, (g) Dral and (h) TaqI. The restriction fragments were resolved by *in silico* electrophoresis through 3 % agarose gel. MW, φX174 DNA-HaeIII digest.
water controls for this process. These results indicated that there are phytoplasmas in diseased P. clematidea plants which may be associated with phyllody disease.

We used iPhyClassifier, the interactive online tool [11], to perform sequence similarity analyses. This process revealed that the 16S rRNA gene sequence (KY568717)
shares 99.5 % similarity with that of the ‘Ca. Phytoplasma australasiae’ reference strain (Y10097). So, the PCP phytoplasma is a ‘Ca. Phytoplasma australasiae’-related strain.

The virtual RFLP pattern derived from the query 16S rRNA gene R16F2n/R16R2 fragment is different from the reference patterns of all previously established 16Sr groups/subgroups. The most similar RFLP pattern is the reference pattern of the 16Sr group II, subgroup M (HG792252), with a similarity coefficient (F) of 0.94 (Table 1), which is less than 0.97, the cutoff value for the delineation of new phytoplasmas 16Sr subgroup lineages [13]. The value of the similarity coefficient (F=0.94) therefore allowed the classification of the PCP phytoplasma as a representative of a new subgroup of 16SrII, 16SrII-V. We also compared virtual RFLP patterns and identified the key enzymes that distinguish the 16SrII-V pattern from all subgroups within the group 16SrII (Fig. 1a). Its virtual MseI RFLP pattern distinguished PCP phytoplasma from all other subgroups in group 16SrII (Fig. 1b). The virtual RFLP patterns of Alul, Bfai, HaeIII and HinfI distinguished PCP phytoplasma from other subgroups in group 16SrII except 16SrII-M (Fig. 1c–i). The virtual DraI RFLP pattern distinguished PCP phytoplasma from other subgroups in group 16SrII except 16SrII-M and 16SrII-R (Fig. 1g). Furthermore, as the most close relationship is between 16SrII-M and 16SrII-V, we analysed the differences between these subgroups. These subgroups can also be differentiated by TaqI (Fig. 1h).

A phylogenetic tree was reconstructed using the minimum evolution approach of the MEGA5 program [12]. Phylogenetic analysis clearly demonstrated that PCP phytoplasma is clustered in the 16SrII group but emerges from a branch that is different from those of the other phytoplasmas of this group (Fig. 2).

Phytoplasmas have been previously reported in P. clematidea in Hainan province, PR China and have been associated with symptoms of witches’ broom, axillary shoot proliferation and dwarfing [8]. Here, a new 16Sr subgroup, 16SrII-V, phytoplasma found in association with PCP disease is reported. To our knowledge, this is the first record of a phytoplasma associated with phylloxy disease of P. clematidea anywhere in the world.

The peanut witches’ broom phytoplasma group (16SrII) is one of the most widely distributed strains worldwide. Twenty-one 16SrII subgroups (A–U) have been described (Table 1) [3, 5, 14–23]. Moreover, the discovery of the 16SrII-V subgroup, described herein, expands our knowledge of the genetic diversity within the 16SrII group.

This work contributes to increasing the knowledge about the diversity of phytoplasmas present in tropical regions of China and also supports the concept that a unique ecology and geographical separation provided favourable conditions for divergence of phytoplasma lineages from other regions of the world.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

References


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