Identification of a novel subgroup 16SrII-U phytoplasma associated with papaya little leaf disease

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Papaya is an important fruit crop cultivated in tropical and subtropical regions. Papaya little leaf (PLL) disease was observed in China. The phytoplasma 16S rRNA gene was detected from symptomatic papaya trees via PCR using phytoplasma universal primers P1/P7 followed by R16F2n/R16R2. No amplification products were obtained from templates of asymptomatic papaya trees. These results indicated a direct association between phytoplasma infection and PLL disease. Comparative and phylogenetic analyses of 16S rRNA gene sequences indicated that the papaya-infecting phytoplasmas under study belonged to the peanut witches’ broom phytoplasma group (16SrII). Genotyping through use of computer-simulated RFLP analysis of 16S rRNA genes and coefficients of RFLP pattern similarities (0.97) reveal that the PLL phytoplasma was placed in a new subgroup. In this article, we describe the molecular characterization of a new phytoplasma associated with PLL disease and propose that the PLL phytoplasma be considered as a novel subgroup, 16SrII-U.

Papaya (Carica papaya L.) is one of the world’s most important fruit crops. Papaya is a rich source of vitamins, antioxidants and fibre and is therefore considered to be a good source of nutrition (Wall & Tripathi, 2014). China is the world’s main producer of papaya, predominantly in Hainan province. Papaya is also known as the king of South of the Five Ridges fruit in China.

Phytoplasmas are unicellular prokaryotes associated with diseases in more than 1000 plant species worldwide (Maejima et al., 2014; Marcone, 2014). They are transmitted by insect vectors (leafhoppers, planthoppers and psyllids) and induce disease symptoms such as yellowing, witches’ broom, phyllody, sterility of flowers, generalized stunting and phloem necrosis (Bertaccini, 2007; Namba, 2011). Due to their inability to grow in cell-free medium, phytoplasmas are mainly classified based on 16S rRNA gene sequencing (Brown et al., 2007; Firrao et al., 2004, 2005; Wei et al., 2007; Zhao et al., 2009). To date, 37 ‘Candidatus (Ca.) Phytoplasma’ species have been formally described based on 16S rRNA gene sequence phylogeny and/or biological/phytopathological characteristics (Firrao et al., 2004; Harrison et al., 2014). Based on their identification and classification by RFLP analysis of 16S rRNA gene sequences, 32 phytoplasma/16Sr groups and more than 100 subgroups have been identified (Lee et al., 1993, 1998; Nejat et al., 2013).

From 2012 to 2014, we conducted phytoplasma disease surveys mainly on woody plants in the Danzhou campus of Hainan University and surrounding area (nearly 1.2 km²), in Danzhou City, Hainan Province, China. We observed a suspected phytoplasma disease on papaya trees. The objectives of this work were to identify the causal agent associated with the diseased papaya trees and to carry out a molecular characterization of the phytoplasma.

Diseased papaya trees displayed little leaves, foliar chlorosis and axillary shoot proliferation (Fig. S1, available in the online Supplementary Material). Leaf samples were randomly collected from six symptomatic papaya plants displaying typical little leaf symptoms. Similar samples were also collected from healthy plants grown in an insect-proof greenhouse for use as negative controls.

Total nucleic acid was extracted from both infected and healthy papaya trees using a DNA extraction kit (Dneasy
| Strain                                      | GenBank accession no. | Classification 2013 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
|--------------------------------------------|-----------------------|---------------------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| Peanut WB phytoplasma                      | L33765                | 16SrII-A             | 1.00 |
| Ca. Phytoplasma aurantifolia               | U15442                | 16SrII-B             | 0.88 | 1.00 |
| Faba bean phytoplasma                      | X83432                | 16SrII-C             | 0.94 | 0.94 | 1.00 |
| Ca. Phytoplasma australasia                | Y10097                | 16SrII-D             | 0.95 | 0.91 | 0.97 | 1.00 |
| Picris echinata phytoplasma                | Y16393                | 16SrII-E             | 0.90 | 0.85 | 0.91 | 0.94 | 1.00 |
| Cactus WB phytoplasma—YN11                 | EU099556              | 16SrII-F             | 0.91 | 0.93 | 0.97 | 0.94 | 0.88 | 1.00 |
| Cactus WB phytoplasma—YN23                 | EU099568              | 16SrII-G             | 0.91 | 0.91 | 0.97 | 0.94 | 0.88 | 0.94 | 1.00 |
| Cactus WB phytoplasma—YN24                 | EU099569              | 16SrII-H             | 0.90 | 0.92 | 0.96 | 0.93 | 0.87 | 0.93 | 0.95 | 1.00 |
| Cactus WB phytoplasma—YN06                 | EU099551              | 16SrII-I             | 0.90 | 0.89 | 0.95 | 0.92 | 0.87 | 0.92 | 0.91 | 1.00 |
| Cactus WB phytoplasma—YN07                 | EU099552              | 16SrII-J             | 0.88 | 0.88 | 0.94 | 0.91 | 0.85 | 0.91 | 0.91 | 0.90 | 0.89 | 1.00 |
| Cactus WB phytoplasma—YN07                 | EU099572              | 16SrII-K             | 0.88 | 0.88 | 0.94 | 0.91 | 0.85 | 0.91 | 0.91 | 0.90 | 0.89 | 0.88 | 1.00 |
| Cactus WB phytoplasma—YN28                 | EU099546              | 16SrII-L             | 0.87 | 0.87 | 0.92 | 0.90 | 0.86 | 0.89 | 0.88 | 0.88 | 0.87 | 0.87 | 1.00 |
| Tephrosia purpurea WB phytoplasma          | HG72252               | 16SrII-M             | 0.81 | 0.86 | 0.92 | 0.89 | 0.85 | 0.89 | 0.89 | 0.88 | 0.87 | 0.86 | 0.86 | 0.85 | 1.00 |
| Bunchy top symptom phytoplasma—IIN-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 |
| Tabebuia 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 |
| 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 |
| Papaya bunchy top phytoplasma—BTSpHv02-IIA | JF781310              | 16SrII-Q             | 0.97 | 0.86 | 0.91 | 0.92 | 0.87 | 0.88 | 0.88 | 0.88 | 0.87 | 0.86 | 0.86 | 0.84 | 0.84 | 0.90 | 0.89 | 0.92 | 1.00 |
Plant Mini Kit; Qiagen). A nested PCR was used for the detection of phytoplasmas using the universal primers P1/P7 (Deng & Hiruki, 1991; Schneider et al., 1995) followed by R16F2n/R16R2 (Gundersen & Lee, 1996) which amplified a phytoplasma 16S rRNA gene segment (approximately 1.2 kbp). For both 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 60 °C (55 °C for R16F2n/R16R2 primer pair), and 2 min (90 s for R16F2n/R16R2 primer pair) at 72 °C, and a final extension cycle of 10 min at 72 °C. The PCR reaction mixtures contained 1 U Taq DNA polymerase (TaKaRa), 200 mM of each dNTP, 0.4 pmol of each primer, and 1 µl of nucleic acid preparations. One microlitre of diluted (1 : 30) PCR products from the first amplification was used as the template in the second round of PCR. Distilled water was used as the blank control.

Three PCR products from diseased plants were cloned and sequenced. For cloning of the 16S rRNA gene, the target DNA fragments were ligated into the plasmid vector pMD18-T (TaKaRa) and the recombinant plasmid was used to transform Escherichia coli DH5α according to the manufacturer’s instructions. The inserts (three inserts of each PCR product) were sequenced with an automated DNA sequencer (ABI Prism model 3730XL; Applied Biosystems) using primers M13-47 and RV-M (Sangon). Nucleotide sequence data revealed that all clone amplicons were identical. The obtained sequence (1248 nt) designated PLL-DZ01 (papaya little leaf – Dan Zhou 01), as a representative strain, was deposited in GenBank (NCBI) under accession no. KP057205. The sequence was subjected to similarity analysis and computer-simulated RFLP analysis using the iPhyClassifier software (Zhao et al., 2009).

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 MEGA5 software as described by Tamura et al. (2011).

Using nested PCRs, fragments of expected size (1.2 kbp) were obtained from DNA samples of little leaves. No amplification occurred with either primer pair when subjecting samples collected from healthy papaya trees and the water controls to this process. These results indicated a direct association between phytoplasma infection and papaya little leaf (PLL) disease.

We used the interactive online tool iPhyClassifier (Zhao et al., 2009) to perform sequence similarity analyses. This revealed that the 16S rRNA gene sequence (KP057205) shared 99 % similarity with that of the ‘Ca. Phytoplasma aurantiofolia’ reference strain (U15442). The PLL phytoplasma is thus a ‘Ca. Phytoplasma aurantiofolia’-related strain.

The virtual RFLP pattern derived from the query 16S rRNA gene R16F2n/R16R2 fragment was different from the reference patterns of all previously established 16Sr
Fig. 1. Virtual RFLP patterns of the 16S rRNA gene R16F2n/R16R2 fragments of PLL phytoplasma strain DZ01. (a) Virtual RFLP patterns of 17 restriction endonuclease enzymes, (b) BfaI, (c) MseI, (d) TaqI, (e) Sau3A1 and (f) HhaI. The restriction fragments were resolved by in silico electrophoresis through 3% agarose gel. MW, φX174 DNA-HaeIII digest.

Phytoplasmas of many groups, such as 16SrI, 16SrII, 16SrXII, 16SrXIII and 16SrXVII, have been previously reported in papaya worldwide and have been associated with symptoms of mosaicim, yellow clinking, apical necrosis, dieback, bunchy top and axillary shoot proliferation (Acosta et al., 2013; Arocha et al., 2005, 2009; Bekele et al., 2011; Esker et al., 2006; Gibb et al., 1996; Hodgetts et al., 2009; Luis-Pantoja et al., 2015; Melo et al., 2013; Pérez et al., 2010; Rao et al., 2011; Streten & Gibb, 2006; Verma et al., 2012; Walsh et al., 2006; White et al., 1997, 1998). Here, a new 16Sr subgroup, 16SrII-U, phytoplasma in association with PLL disease is reported. To our knowledge, this is the first record of a phytoplasma disease of papaya in China.

The peanut witches’ broom phytoplasma group (16SrII) exhibits high genetic diversity (Cai et al., 2008; Pérez-López et al., 2016; Zhao et al., 2010). At the time of writing, 20 16S rRNA gene subgroups had been recognized in the peanut witches’ broom phytoplasma group. These are 16SrII-A (represented by peanut witches’ broom phytoplasma, L33765) (Gundersen et al., 1994), 16SrII-B (represented by Ca. Phytoplasma aurantifolia, U15442) (Zreik et al., 1995), 16SrII-C (represented by faba bean phyllody, X83432) (Schneider et al., 1995), 16SrII-D (represented by Ca. Phytoplasma australasiae, Y10097) (White et al., 1998), 16SrII-E (represented by Picris echiodes phyllody phytoplasma, Y16393) (Martini et al., 2007; Seemueiller et al., 1998), 16SrII-F (represented by Cactus witches’ broom –

groups/subgroups. The most similar was the reference pattern of the 16Sr group II, subgroup A (L33765), with a similarity coefficient (F) of 0.97 (Table 1). The value of the similarity coefficient for the delineation of new phytoplasma 16Sr subgroup lineages (F=0.97; Wei et al., 2007) allowed the classification of the PLL phytoplasma as a representative of a new subgroup of 16SrII, 16SrII-U. We also compared virtual RFLP patterns and identified the key enzymes that distinguish the 16SrII-U pattern from all subgroups within group 16SrII (Fig. 1a). The virtual BfaI RFLP pattern distinguished the PLL phytoplasma from all other subgroups in group 16SrII (Fig. 1b). The virtual MseI RFLP pattern distinguished the PLL phytoplasma from other subgroups in group 16SrII except 16SrII-A, -D, -E and -Q (Fig. 1c); the virtual TaqI RFLP pattern distinguished the PLL phytoplasma from other subgroups in group 16SrII except 16SrII-A, -P and -Q (Fig. 1d). As the closest relationship was found to 16SrII-A, -P, -Q and -U, we analysed the differences among these subgroups and found that they could be differentiated from 16SrII-P by Sau3A1 (Fig. 1e) and from 16SrII-Q by HhaI (Fig. 1f).

A phylogenetic tree was reconstructed using the minimum-evolution approach of the MEGA5 program (Tamura et al., 2011). Phylogenetic analysis clearly demonstrated that the PLL phytoplasma clustered in the 16SrII group but emerges from a branch that is different from those of the other phytoplasmas of this group (Fig. 2).
YN11, EU099556) (Cai et al., 2008), 16SrII-G (represented by Cactus witches’ broom – YN23, EU099568) (Cai et al., 2008), 16SrII-H (represented by Cactus witches’ broom – YN24, EU099569) (Cai et al., 2008), 16SrII-J (represented by Cactus witches’ broom – YN06, EU099551) (Cai et al., 2008), 16SrII-K (represented by Cactus witches’ broom – YN28, EU099572) (Cai et al., 2008), 16SrII-L (represented by Cuban papaya phytoplasma, DQ286948) (Pérez-López et al., 2016), 16SrII-P (represented by Cuban papaya phytoplasma, DQ286948) (Pérez-López et al., 2016), 16SrII-N (represented by papaya bunchy top symptom phytoplasma – IIN-LT, JF781309) (Acosta et al., 2013), 16SrII-O (represented by Tabebuia pentaphylla phytoplasma, EF647744) (Pérez-López et al., 2016), 16SrII-P (represented by Cuban papaya phytoplasma, DQ286948) (Pérez-López et al., 2016), 16SrII-Q (represented by Peanut WB phytoplasma JF781310 16SrII-Q) (Pérez-López et al., 2016).
(Pérez-López et al., 2016), 16SrII–Q (represented by papaya bunchy top phytoplasma – BTSpHav02–IIA, JF781310) (Pérez-López et al., 2016), 16SrII–R (represented by Echinopsis sp. yellow leaf phytoplasma, DQ535900) (Pérez-López et al., 2016), 16SrII–S (represented by Amaranthus hypochondriacus phytoplasma–52A, FJ357164) (Pérez-López et al., 2016) and 16SrII–T (represented by tomatillo WB phytoplasma, EU125185) (Pérez-López et al., 2016). Furthermore, the discovery of the 16SrII–U subgroup, described herein, expands our knowledge of the genetic diversity within the 16SrII group.

Given that the 16SrII–U subgroup has not yet been reported in another host, and considering the similarities found in sequences with 16SrII–D, -N, -P and 16SrII–Q subgroup isolates from papaya trees in Australia and Cuba, PLL appears to prevail in papaya trees; however, the assessment of weeds and/or other surrounding crop species is necessary to identify vector insects and confirm this. Study of the diversity of PLL phytoplasma isolates from different regions and the search for their vectors could reveal the behaviour of this pathosystem at a deeper level, thus improving the management strategies of the disease and preventing its spread to unaffected areas.

In summary, we describe the molecular characterization of a new phytoplasma from the tropical area of China associated with a PLL disease and propose that this phytoplasma be considered as a novel ‘Ca. Phytoplasma’ taxon, 16SrII–U. The findings from this study expand current knowledge regarding the genetic diversity of the peanut witches’ broom phytoplasma group and the multiplicity of phytoplasmas affecting plants in China.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 31201492), the Fundamental Research Funds for Environment and Plant Protection Institute, CATAS (Nos. 2012hs1j001 and 2015hs1j006).

References


International Journal of Systematic and Evolutionary Microbiology 66
Phytoplasmas: a century of pioneering research.


