SHORT COMMUNICATION

IDENTIFICATION OF A PHYTOPLASMA ASSOCIATED WITH CHERRY VIRESCENCE IN CHINA

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SUMMARY

During a field survey in 2007, sweet cherry (*Prunus avium* L.) trees with symptom of virescence were found in Yantai, Shandong province of China. Total DNA extracted from symptomatic and symptomless trees was subjected to nested PCR with universal primers for phytoplasmal 16S rRNA gene. A specific fragment of approximately 1.2 kb in length was amplified from diseased but not from healthy samples. Phylogenetic analysis of the 16S rRNA gene sequence showed that the phytoplasma isolate associated with cherry virescence in Yantai (ChV-YT), belongs to '*Candidatus* Phytoplasma asteris' (16SrI group). Phylogenetic and computer simulated RFLP analysis of 16S rRNA gene revealed that ChV-YT represents a new subgroup in the aster yellows group, tentatively designated as 16SrI-S. This is the first report of a phytoplasma associated with cherry virescence in China.

Key words: 16SrI group, computer simulated Phytoplasma RFLP analysis, Nested PCR, *Prunus avium*.

Cherry (*Prunus avium* L.), one of the main fruit trees in the world, according to FAO estimates in 2007 had a total world production next to two million tons. However, the yield and quality of cherry fruits are severely affected by several phytoplasmas. In America, cherry buckskin (or western X-disease; 16SrIII group) occurrs in several US states and almost destroyed the cherry industry in some areas of California (Van Steenwyk *et al*., 1995; Gundersen *et al*., 1996a). In Europe, cherry decline and Moliere's disease associated with phytoplasmas of groups 16SrI (Paltrinieri *et al*., 2001; Varga *et al*., 2001; Navratil *et al*., 2001; Valiunas *et al*., 2009a), 16SrI-II (Paltrinieri *et al*., 2001, 2008; Landi *et al*., 2007; Valiunas *et al*., 2009b), 16SrV (Paltrinieri *et al*., 2008), 16SrX (Lee *et al*., 1998; Paltrinieri *et al*., 2001; Varga *et al*., 2001; Fialová *et al*., 2004; Laviña *et al*., 2004; Cieslinska and Morgas, 2011), and 16SrXII (Schneider *et al*., 1993; Marcone *et al*., 1999; Paltrinieri *et al*., 2001, 2008; Valiunas *et al*., 2009a) are disastrous for cherry production. Finally, cherry fasciated and lethal yellows, associated with phytoplasmas of 16SrVII-A (Li *et al*., 1997) and 16SrV-B (Lee *et al*., 1995; Zhu *et al*., 1998), respectively, were reported from China.

During field surveys of more than 100 Chinese orchards in 2007, cherry trees (cv. Hongdeng) showing virescence symptoms were observed in Yantai (Shandong province). The main symptoms of diseased trees were flower virescence, decline, short internodes, and sterility. Petals exhibited different degrees of greening, some blossoms showed a light green hue in the normally white petals, while other blossoms were entirely green, and some acquired a leaf-like appearance (Fig. 1). These symptoms occurred only on some shoots so that the flowers of a small branch turned completely green, while those of a neighboring branch were normal. Symptomatic branches did not produce fruit.

Symptomatic scions were grafted onto 10 healthy trees of cherry cv. Hongdeng. After two years, virescence appeared in some flowers of four trees, indicating that the disease is transmissible.

Petals and leaf samples were collected from three trees with virescence symptom and two without symptom. Total DNA was extracted from fresh midribs and virescent petals according to Qi *et al*. (2004). Primer pairs R16mF2/ R16mR1 (Lee *et al*., 1993) followed by R16F2/R16R2 (Gundersen and Lee, 1996b) were used to amplify the 16S rRNA gene with nested PCR. The constituents of reaction mixtures and the program utilized were basically the same of our previous report (Gao *et al*., 2008), except that the annealing temperature in the first amplification stage was changed to 55°C. Amplicon were cloned into pMD18-T vector and custom sequenced (Shanghai Biosune Biotechnology, China). At least two clones from separate PCR reactions were sequenced for each sample.

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Table 1. Similarity coefficients derived from analysis of virtual RFLP patterns.

Serial	Strain/ Accession number	1	2	3	4	5	6	7	8	9	10	11
1	16SrI-A/AY389828	1.00										
2	16SrI-B/M30790	0.92	1.00									
3	16SrI-C/AF222065	0.91	0.93	1.00								
4	16SrI-D/AY265206	0.91	0.97	0.90	1.00							
5	16SrI-E/ Y265213	0.91	0.93	0.92	0.90	1.00						
6	16SrI-F/AY265211	0.86	0.88	0.87	0.85	0.87	1.00					
7	16SrI-K/U96616	0.86	0.69	0.85	0.85	0.90	0.90	1.00				
8	16SrI-O/ F268405	0.86	0.87	0.80	0.85	0.80	0.74	0.80	1.00			
9	16SrI-P/AF503568	0.92	0.94	0.93	0.91	0.93	0.94	0.92	0.80	1.00		
10	16SrI-Q/AY034089	0.84	0.92	0.89	0.89	0.85	0.86	0.78	0.80	0.86	1.00	
11	16SrI-S/M148153 (ChV –YT)	0.85	0.91	0.87	0.88	0.85	0.79	0.80	0.82	0.85	0.83	1.00

The similarity coefficient of 16S rRNA gene sequences was calculated automatically by Perl program according to Wei *et al*. (2008).

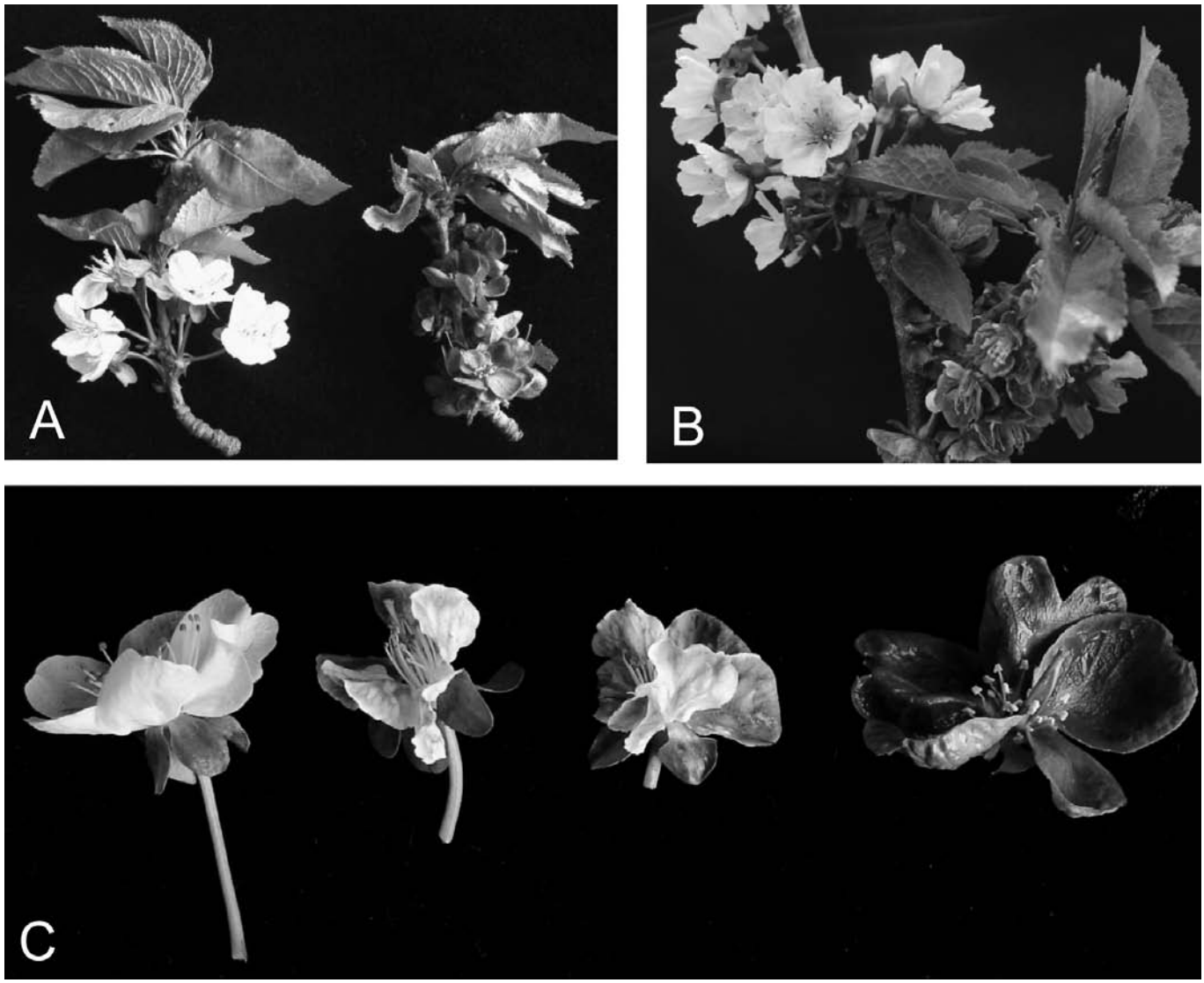

Fig. 1. Symptoms of cherry virescence. A. Branches of healthy (left) and infected (right) sweet cherry trees. B. Symptomatic and symptomless flowers on the same branch of a phytoplasma-infected sweet cherry tree. C. Flowers from a phytoplasma-infected tree (healthy flower on the left).

A fragment 1,236 bp in length was amplified from virescent samples with universal primers for the 16S rRNA gene, but not from healthy cherry trees. Sequences from each clone from the three samples were identical and deposited in GenBank (accession No. HQ148153). The potential phytoplasma associated with cherry virescence was designated as ChV-YT.

Nucleotide sequences similarity calculation and multiple sequences alignment conducted using DNAStar (LASERGENE software) showed that the 16S rRNA gene sequence of ChV-YT had similarity of 98.9-99.4% with phytoplasmas of the aster yellows group (16SrI group), and of 91.9-92.5% and 90.3-90.5% with those of the 16SrX and 16SrV groups, respectively. It was also found that ChV-YT had similarity of 99.2% with both cherry little leaf phytoplasma (16SrI-Q; accession No. AY034089) and paulownia witches'-broom phytoplasma (16SrI-D; accession No. AY265206), and the highest similarity (99.4%) with the OAY reference strain of *Candidatus* Phytoplasma asteris (*Ca.* P. asteris) (16Sr I-B; accession No. M30790).

A phylogenetic tree was constructed with sequences of the 16S rRNA gene, using the neighbour joining and maximum parsimony methods implemented in MEGA 4.0 (Tamura *et al.*, 2007) and bootstrap analysis with 1000 replicates for evaluating the significance of the internal branches. In this tree, ChV-YT formed a subbranch with aster yellows phytoplasma (AF268405; 16SrI-O), cherry little leaf phytoplasma (AY034089; 16SI-Q) and cherry proliferation phytoplasma (FJ231729; 16SI-B) (Fig. 2).

Furthermore, the 16S rRNA gene sequences of ChV-

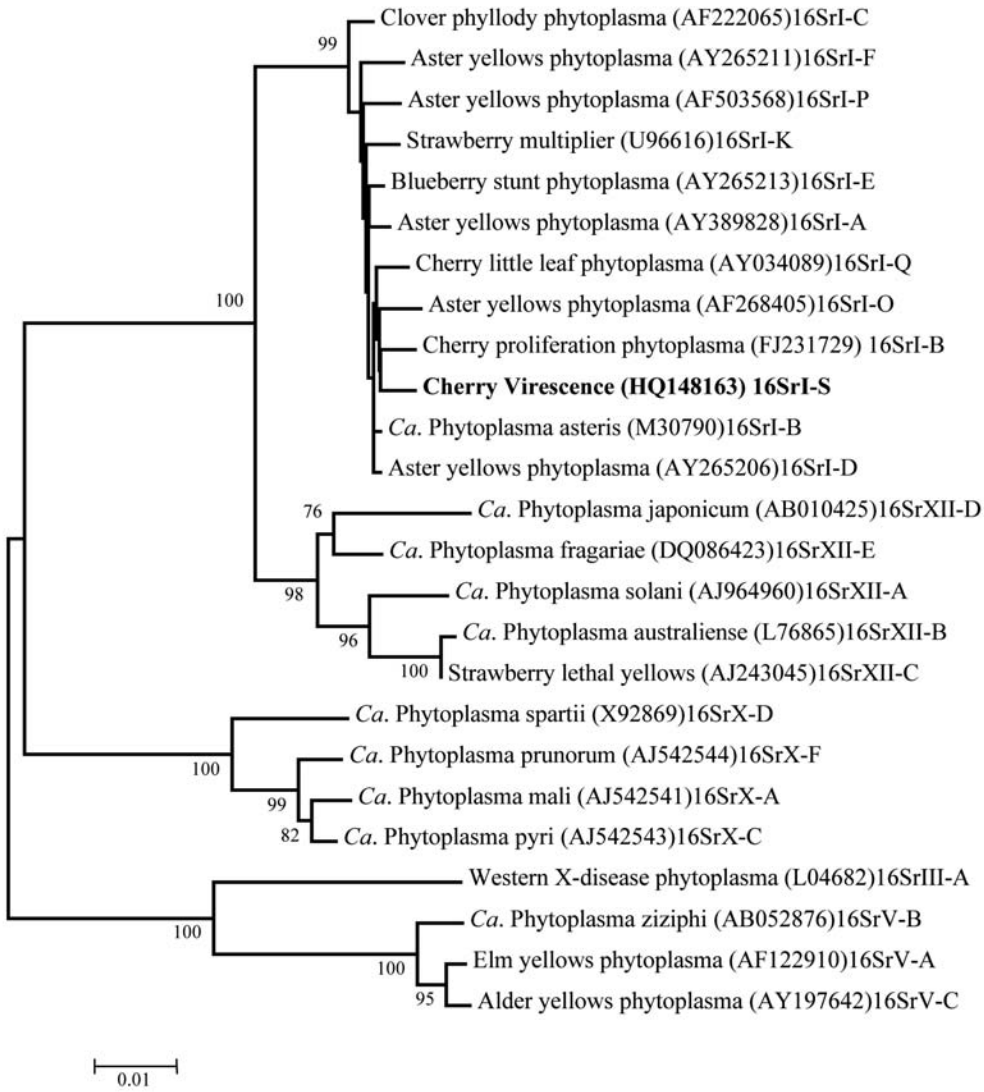

Fig. 2. Phylogenetic tree based on 16S rRNA gene of the phytoplasma associated with cherry virescence and other 24 phytoplasmal strains. The tree was constructed by neighbour-joining analysis (MEGA, version 4.0) obtained from aligning partial nucleotide sequence of the 16S rRNA from the phytoplasma infecting sweet cherry trees and phytoplasmal reference strains available in GenBank. The data were replicated 1000 times and the bootstrap values >70% are given at the nodes of the phylogenetic tree. The scale bar represents a distance of 0.02 substitutions per nucleotide site.

YT and other 10 reference strains of the 16SrI group were analyzed by RFLP pattern comparison and the similarity coefficient calculated with Perl program (Wei *et al*., 2008). Results showed that ChV-YT shared similarity coefficient lower than 0.97 with all subgroups members (Table 1). Therefore, the ChV-YT isolate of the phytoplasma associated with cherry virescence in China belongs in the aster yellows group, representing a new subgroup, which is tentatively designated as 16SrI-S. The same results were obtained when the interactive online phytoplasma classification tool, iPhyClassifier (Zhao *et al*., 2009) was used to analyze the sequence (not shown).

The similarity and phylogenetic analyses of 16S rRNA gene sequence play a pivotal role in the differentiation of groups, but are not effective enough to differentiate some subgroups, including subgroups B and D of the 16SrI group. Thus, analysis of elongation factor EF-Tu gene (Marcone *et al*., 2000), SecY gene (Lee *et al*., 2006) and ribosomal protein gene (Gao *et al*., 2008) can help to elucidate the taxonomic status of a phytoplasmal strain.

In our study, the phytoplasma associated with cherry virescence disease was characterized and classified as a new subgroup, 16SrI-S, of '*Ca*. P. asteris' group based on the results of phylogenetic and RFLP analyses of the 16S rRNA gene. This is the first report on the occurrence of cherry virescence in China. However, the low incidence of the disease suggests that affected trees might have been introduced from other countries through commercial trading. To prevent disease spreading symptomatic cherry trees were eliminated. The possible occurrence of cherry virescence in other regions of China is now under investigation.

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