

LESS COMMON PHYTOPLASMAS INFECTING STONE FRUIT TREES

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Abstract: *Prunus* species plants can be infected by eight '*Candidatus* Phytoplasma' ('*Ca. P.*') species classified to eight distinctive taxonomic groups: '*Ca. P. prunorum*' (16SrX-B), '*Ca. P. mali*' (16SrX-A), '*Ca. P. pyri*' (16SrX-C), '*Ca. P. asteris*' (16SrI), '*Ca. P. aurantifolia*' (16SrII), '*Ca. P. ziziphi*' (16SrV), '*Ca. P. fraxini*' (16SrVII), '*Ca. P. phoenicium*' (16SrIX) and two potentially new species: '*Ca. P. pruni*' (16SrIII) and '*Ca. P. solani*' (16SrXII). These agents occur incidentally in orchards and their impact on stone fruit production is lower than '*Candidatus* Phytoplasma prunorum'. Hosts, geographic distribution, symptoms and insect vectors of these '*Ca. P.*' species, methods of their identification, and control management are reviewed.

Key words: '*Candidatus* Phytoplasma' species, geographic distribution, hosts, symptoms, insect vectors, detection, identification

Phytoplasmas are wall-less plant pathogenic bacteria classified in the class *Mollicutes* transmitted from plant to plant by grafting and phloem-feeding insects (Davis and Lee 2000). In 2004, the name phytoplasma was adopted and is currently at *Candidatus* status (IRPCM 2004). Classification of phytoplasmas is mainly based on the nucleotide sequence of the 16S rRNA gene (Lim *et al.* 1989; Gundersen *et al.* 1994; Seemüller *et al.* 1994). Restriction Fragment Length Polymorphism (RFLP) analysis of this genome fragment made it possible to classify phytoplasmas into twenty-eight groups (16Sr groups), and more than 50 subgroups (Lee *et al.* 1998b; Marcone *et al.* 2000; Wei *et al.* 2007).

European stone fruit yellows (ESFY) disease is one of the most devastating, quarantine phytoplasma disease leading to economic damage in *Prunus* species throughout Europe and Asia Minor (Carraro and Osler 2003; Marcone *et al.* 2010). '*Candidatus* Phytoplasma prunorum' ('*Ca. P. prunorum*', 16SrX-B) and its association with ESFY was reported in the author's previous paper (Cieślińska *in press*). Stone fruit trees can also be infected by phytoplasmas classified to groups other than the 16SrX groups: aster yellows (16SrI), peanut witches'-broom (16SrII), X disease (16SrIII), elm yellows (16SrV), ash yellows (16SrVII), pigeon pea witches' broom (16SrIX), and stolbur (16SrXII), as well as phytoplasmas belonging to the two other subgroups (16SrX-A and -B) of the apple proliferation group. Some of these agents are distributed only in Europe while others (phytoplasmas from 16SrIII, V, VII and XII groups) were reported both in Europe and other continents.

Aster yellows group (16SrI, '*Candidatus* Phytoplasma asteris')

The occurrence of '*Candidatus* Phytoplasma asteris' (Lee *et al.* 2004a) was reported in apricot, plum, nectarine, and Japanese plum in Italy (Lee *et al.* 1998a). This agent caused apricot chlorotic leaf roll in apricot in Spain (Schneider *et al.* 1993) and was also detected in peach, apricot, European plum, sour cherry, sweet cherry, blackthorn and almond trees in the Czech Republic (Navrátil *et al.* 2001; Fialová *et al.* 2004). Navrátil *et al.* (2001) showed that in the Czech Republic sweet cherry showing stunting, leaf rolling, and yellowing and sour cherry trees with small leaves, reduced vigor, and die-back were mainly infected by '*Ca. P. asteris*'. This agent was also associated with cherry little leaf disease of sour cherry trees in Lithuania showing shoot proliferation, small leaves, and decline symptoms and was classified to a new subgroup 16SrI-Q (Valiūnas *et al.* 2009a). It was found that sour cherry with bunchy little leaf growths and leaf drop was infected by phytoplasma related to clover phyllody (formerly 16SrI-C) (Jomantienne *et al.* 2011). Plum exhibiting symptoms of witches'-broom, shoot proliferation and abnormally small leaves, and sour cherry in Lithuania (Valiūnas *et al.* 2007) as well as mahaleb in Hungary (Varga *et al.* 2001) were infected by phytoplasma strains included into the 16SrI-B subgroup. Strains in the aster yellows group share $\geq 97\%$ similarity in their 16S rDNA sequences (Lee *et al.* 2004a). Leafhopper species: *Macrostelles*, *Euscelis*, *Scaphytopius*, and *Aphrodes* are the major vectors of '*Ca. P. asteris*' (Chiykowski 1991; Lee *et al.* 2004a).

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For diagnosis of 'Ca. P. asteris' using PCR, several specific primer pairs based on the 16S rDNA sequence of aster yellows phytoplasmas (16SrI) were designed so far. Among them, R16(I)F1/R1 (Lee *et al.* 1994) is routinely used as a primer pair in nested PCR followed P1/P7. Differentiation of 'Ca. P. asteris' strains after amplification of 16S rDNA fragment by nested PCR with universal primers P1/P7 followed by R16F2n/R16R2, is possible by using *AluI*, *BfaI*, *HaeIII*, *HhaI*, *Hinfl*, *HpaII*, *KpnI*, *MseI*, *RsaI*, *Sau3AI*, and *TaqI* restriction enzymes (Lee *et al.* 1998b; Marcone *et al.* 2000; Valiūnas *et al.* 2007). Virtual RFLP analysis of the 16S rRNA gene for 16 strains of 'Ca. P. asteris' belonging to different subgroups, resulted in two different restriction profiles after digestion with *HaeIII* enzyme, and three various patterns when *RsaI* endonuclease was used Valiūnas *et al.* (2009a). Profiles after digestion with *HaeIII* enzyme of 16S rDNA fragment of phytoplasmas causing ChLL (cherry little leaf) and ACLR (apricot chlorotic leaf roll) differed from each other. RFLP result and phylogenetic analysis allowed the classification of the ChLL phytoplasma strain to the new established subgroup 16SrI-Q. Apart from 16S rRNA, several other genes were analyzed using PCR/RFLP and sequence analysis to identify molecular characterization of aster yellows phytoplasma infecting *Prunus* sp. plants: *tuf* (encodes the elongation factor) from apricot showing apricot chlorotic leafroll symptoms (Schneider *et al.* 1993); *rp* (encodes ribosomal proteins) operon genes for phytoplasma inducing cherry little leaf symptoms (16SrI-Q) in sour cherry from Lithuania (Lee *et al.* 2004a). Based on collective RFLP patterns of 16S rDNA and *rp* gene, the phytoplasma from sour cherry exhibiting bunchy little leaf growths was classified in subgroup 16SrI-R and new *rp* subgroup *rpI-O* (Jomantiene *et al.* 2011).

Peanut witches'-broom group (16SrII, 'Candidatus Phytoplasma aurantifolia')

Zreik *et al.* (1995) showed that the sweet cherry trees growing in central regions of Iran, were infected with two different phytoplasmas. 'Ca. P. asteris' was detected in the trees with leaf roll, and witches' broom symptoms. However, sequence analysis of 16S rRNA gene and 16S-23S intergenic spacer region indicated that sweet cherry trees with little leaf and rosetting symptoms were infected with phytoplasma related to 'Candidatus Phytoplasma aurantifolia' classified to the peanut witches'-broom group (16SrII) (Zirak *et al.* 2010). The role of the insect vectors in the outbreak of diseases in Iran is still unknown.

Virtual RFLP analysis of 16S rDNA with 17 restriction enzymes was used for differentiation of phytoplasmas from peanut witches'-broom group (16SrII) on 12 subgroups clustered in two 'Ca. Phytoplasma' species: 'Ca. P. aurantifolia' and 'Ca. P. australasia' (Cai *et al.* 2008).

X-disease group (16SrIII, 'Candidatus Phytoplasma pruni')

Phytoplasmas classified to the X-disease group (16SrIII), with the suggested name: 'Candidatus Phytoplasma pruni' (IRPCM 2004), were identified in the USA in peach, sour cherry, and sweet cherry as well as in chokecherry (*Prunus virginiana* L.) known as a reservoir host of this agent (Granett and Gilmer 1971; Lee *et al.*

1992; Kirkpatrick *et al.* 1995). The phytoplasma was also detected in Italy, in declining cherry trees imported from California (Paltrinieri *et al.* 2001; Landi *et al.* 2007). The symptoms of sparse canopies, small leaves and small, pointed fruits on sweet cherry, mazzard (*Prunus avium*), Stockton Morello (*Cerasus vulgaris*) and Colt (*P. avium* × *P. pseudocerasus*) trees grown in California were caused by phytoplasmas from the X disease group (Uyemoto and Luhn 2006). In contrast, this agent induced stem pits and grooves at the scion-rootstocks junction, and declining of the sweet cherry trees grafted on *P. mahaleb* (Uyemoto 1989). Peach infected with X-disease phytoplasma developed leaf symptoms consisting of chlorotic spots that turned necrotic, drop out leaving a shot-hole appearance, limb and scaffold branch dieback, poor fruit set and tasteless flesh. All these symptoms were found in chronically infected trees (Uyemoto and Luhn 2006). Sour cherry and sweet cherry infected by this agent in Lithuania developed leaf reddening and premature leaf drop, proliferation of branches and non-seasonal flowering (Valiūnas *et al.* 2009b). X-disease phytoplasma ('Ca. P. pruni') is transmitted by many leafhopper species (Lee *et al.* 2000). Over ten species of these vectors were reported in the USA where these agents cause high economic losses in *Prunus* sp. orchards. Six species of leafhoppers: *Colladonus*, *Fieberiella*, *Gyponoma*, *Norvellina*, *Paraphlepsius* and *Scaphytopius* were identified in Connecticut as the vectors of 'Ca. P. pruni'. It was evidenced that *Paraphlepsius irroratus* (Say) and other leafhopper species transmitted peach X-disease phytoplasma from chokecherry to peach (Rosenberger and Jones 1978).

Based on the 16S rRNA gene sequence Lee *et al.* (1994) designed primers R16(III)F2/R1 for specific amplification of phytoplasmas from X-disease group (16SrIII). RFLP analysis using *MseI*, *HhaI*, *HpaII*, *BfaI* and *Sau3AI* restriction enzymes resulted in distinguish of phytoplasmas associated with X-disease of *Prunus* sp. from the reference strains CX and WX (16SrIII-A) and allowed to classify X-disease phytoplasmas into eight subgroups, A-H (Lee *et al.* 1998b). Virtual RFLP analysis allowed for the division of the phytoplasmas causing X-disease into 19 subgroups (Zhao *et al.* 2009). *rp* (encodes ribosomal proteins) operon genes were also analyzed using PCR/RFLP and sequence analysis to identify and discriminate the

X-disease phytoplasmas in peach and chokecherry (Gundersen *et al.* 1996). Southern blot hybridization was a useful method for differentiation of phytoplasmas belonging to the X-disease group (Lee *et al.* 1992).

Significant reduction of X-disease symptoms in sweet cherry orchards were achieved by cutting down the symptomatic trees and spraying with diazinon to prevent the infected leafhoppers from leaving (Uyemoto *et al.* 1998). The removal of infected plants and phytoplasma reservoir hosts is the most effective method in orchards. In California, eradication of cherry trees infected with X-disease protected the orchards from spreading the phytoplasma (Uyemoto *et al.* 1998). Treating cherry and peach trees with a tetracycline antibiotic, which is bacteriostatic to phytoplasmas and inhibit their growth (Davis *et al.* 1968), significantly reduced X disease in orchards (Lee *et al.* 1987). Injection of phytoplasma-infected peach

trees with an OTC hydrochloride solution resulted in a remission of symptoms the following year (Thakur *et al.* 1998). It is important to point out, that without continuous use of the antibiotic, disease symptoms reappear.

Elm yellows group (16SrV, '*Candidatus Phytoplasma ziziphi*')

'*Candidatus Phytoplasma ziziphi*' (Jung *et al.* 2003) was identified in sour cherry trees showing cherry lethal yellows (CLY) symptoms in China and northern Italy (Lee *et al.* 1995b; Zhu *et al.* 1998) as well as in apricot and cherry plum with yellow, small and upward curled leaves in China (Yue *et al.* 2009; Hong *et al.* 2011). This agent was also associated with peach yellows (PY) in India. The infected trees showed chlorosis, upward rolling and occasional red spotting of the leaves (Thakur *et al.* 1998). The presence of phytoplasmas related to the elm yellows group was detected in several stone fruit trees grown in experimental orchards in Poland (Cieślińska *et al.* 2004). In nature, the phytoplasmas classified to the 16SrV group are transmitted by different insect vectors (Lee *et al.* 2004b).

These agents share a 98.6–99.9% similarity, thus they appear to be homogenous on the basis of 16S rDNA sequences (Lee *et al.* 1998b). Primers R16(V)F1/R1 (Lee *et al.* 1994) can be used for specific amplification of 16S rDNA fragment of phytoplasmas belonging to the 16SrV group (elm yellows group): '*Ca. P. ulmi*' (16SrV-A), '*Ca. P. ziziphi*' (16SrV-B), '*Ca. P. vitis*' (16SrV-C). Phytoplasmas classified to subgroups A and B can be distinguished by using primers specific to subgroup 16SrV-B (Zhu *et al.* 1998) or by digestion of the PCR products amplified using R16F2n/R16R2 with *RsaI* and *HpaII* endonucleases. In turn, *BfaI* enzyme distinguishes phytoplasmas of subgroups A and B from the agents classified to the 16SrV-C subgroup (Lee *et al.* 1998b). On the basis of RFLP analysis of 16S ribosomal RNA gene, the causal agent of peach yellows was classified to subgroup B of the 16SrV group beside cherry lethal yellows and jujube witches'-broom (JWB) phytoplasmas (Thakur *et al.* 1998). Lee *et al.* (2004b) recommended using the elm yellows-group specific ribosomal protein primer pairs rp(V) F1/rpR1 followed by rp(V)F1A/rp(V)R1A1 for amplification of the *rp* gene operon of phytoplasmas classified to the elm yellows group (16SrV). *rp* and *secY* (encodes protein involved in protein secretion mechanism from bacteria) operons were used for molecular characterization of '*Ca. P. ziziphi*' from sweet cherry with cherry lethal yellows symptoms (Zhu *et al.* 1998; Lee *et al.* 2004b) and from peach with peach yellow symptoms (Thakur *et al.* 1998). Members of 16SrV-B associated with different plant hosts, were subdivided on three ribosomal protein (*rp*) subgroups and three *secY* subgroups (Lee *et al.* 2004b). The other strains belonging to 16SrV could be differentiated from '*Ca. P. ulmi*' (16SrV-A) by restriction analysis of 16S rDNA with *RsaI* and *BfaI* enzymes or using *Tsp509I* or *MseI* for digestion of *rpl22-rps3* ribosomal protein gene (Firrao *et al.* 2005).

Ash yellows group (16SrVII, '*Candidatus Phytoplasma fraxini*')

Prunus sp. trees can be also infected by '*Candidatus Phytoplasma fraxini*'. The occurrence of a 16SrVII-related

phytoplasma strain was reported in China in sweet cherry exhibiting fasciation (Li *et al.* 1997). Paltrinieri *et al.* (2003) based on molecular biology methods, identified this phytoplasma in peach, in southern Italy. In Canadian Clonal Genbank, five accessions of peach trees showing decline, leaf reddening, yellowing, shortening of internodes, witches' broom and reduced fruit size were positively tested for '*Ca. P. fraxini*' (Zunnoon-Khan *et al.* 2010). It was shown that the phytoplasma detected in peach trees, in Canada, shared 99% 16S rDNA sequence identity with '*Ca. P. fraxini*' (16SrVII). RFLP profiles after digestion of R16F2n/R16R2-amplified products with *AluI*, *RsaI* and *MseI* were similar to the patterns of the 16SrVII-A phytoplasma subgroup. Restriction enzymes *HhaI* and *TaqI* differentiate '*Ca. P. fraxini*' from the phytoplasmas classified to most of the other 16Sr groups (Lee *et al.* 1998b).

Pigeon pea witches'-broom group (16SrIX, '*Candidatus Phytoplasma phoenicium*')

'*Candidatus Phytoplasma phoenicium*' causing almond witches'-broom (Verdin *et al.* 2003) was identified in almond, peach, and nectarine trees in the Middle East (Abou-Jawdah *et al.* 2003). The phytoplasma induced early flowering, stunted growth, leaf rosetting, dieback, off-season growth, proliferation of slender shoots, and witches'-brooms of infected almond trees. The disease spread very fast in orchards. Peach and nectarine seedlings grown near an infected almond trees showed proliferation and/or leaf roll.

The primer pair, AlmF1/AlmR1 was defined by Verdin *et al.* (2003) for the specific amplification of the 16S rDNA fragment of '*Ca. P. phoenicium*'. It was possible to differ this agent from the other phytoplasmas classified to 16SrIX group using RFLP with *TruI* i *TaqI* restriction enzymes (Al-Saady *et al.* 2008).

Apple proliferation group (16SrX, '*Candidatus Phytoplasma mali*' and '*Candidatus Phytoplasma pyri*')

'*Ca. P. mali*' (16SrX-A) is the causal agent of apple proliferation disease but its host range is not limited only to *Malus* species. Occurrence of '*Ca. P. mali*' was described in Japanese plum in Italy (Lee *et al.* 1995a), peach and sweet cherry trees in Italy (Paltrinieri *et al.* 2001), sweet cherry in the Czech Republic (Navrátil *et al.* 2001), sweet cherry, apricot and plum in Slovenia (Mehle *et al.* 2007) as well as in nectarine in Poland (Cieślińska and Morgaś 2011). The pathogen was identified in symptomatic as well asymptomatic trees. Some of the infected peach and plum bloomed later than healthy trees and showed weak vigour. Sweet cherry showed stunting, leaf rolling, yellowing, wilting, floral and phloem necrosis and declining. Some apricot positive tested for '*Ca. P. mali*' exposed stem necrosis and leaf wilting, and nectarine showed stunting, chlorotic leaf roll, and premature reddening of the leaves.

In turn, '*Ca. P. pyri*' (16SrX-C), which is essentially associated with pear decline disease, was detected in sweet cherry in Italy (Paltrinieri *et al.* 2001) and Poland (Cieślińska and Morgaś 2011) as well as in peach trees showing symptoms of leaf roll and yellowing in the Czech Republic (Navrátil *et al.* 2001; Fialová *et al.* 2004). Peach yellow leaf roll phytoplasma (PYLR), the disease

of stone fruits in North America, is most closely related to 'Ca. P. pyri' (99.6% identity of 16Sr RNA gene). For this reason, PYLR is regarded as a subtype of 'Ca. P. pyri' (Seemüller and Schneider 2004). PYLR caused major losses in peach, leading to rapid decline of the orchards in the late 1970's (Purcell *et al.* 1981). Its highest incidence was found in California, in peach orchards adjacent to pear orchards. Pear psyllids are the primary vectors of PYLR, and transmit the phytoplasma from pear to peach trees (Purcell *et al.* 1981; Blumquist and Kirkpatrick 2002).

Cacopsylla spp. transmit phytoplasmas from AP group (16SrX) infecting pome and stone fruit trees (Blomquist and Kirkpatrick 2002; Weintraub and Beanland 2006; Weintraub and Wilson 2010).

The phytoplasmas from the apple proliferation group (16SrX) can be distinguished from each other by obtaining unique restriction sites or their absence after digestion of 16S rDNA fragment with *RsaI*, *SspI*, *BsaAI* and *SfeI* enzymes (Seemüller and Schneider 2004). The apple proliferation group-specific primers: R16(X)F1/R1 (Lee *et al.* 1995a) or f01/r01 (Lorenz *et al.* 1995) are also used in the nested PCR for phytoplasmas belonging to the apple proliferation group. If f01/r01 primers are used, the amplification products may be digested by *BsaAI*, *SspI* and *SfeI* endonucleases for differentiation of these three phytoplasmas (Lorenz *et al.* 1995; Carraro *et al.* 1998). Real-time PCR was successfully applied for specific detection and quantification of phytoplasmas from the apple proliferation group in stone fruit trees and insects (Jarausch *et al.* 2004). *Imp*, *pnp*, *aceF* and *secY* markers were analyzed for multilocus sequence typing (MLST) in the 16SrX phytoplasma taxonomic group (Danet *et al.* 2007). Besides PCR/RFLP and real time PCR techniques, Southern blot hybridization assay was also applied for differentiation of phytoplasmas belonging to the apple proliferation group (Kison *et al.* 1997; Kison and Seemüller 2001).

Stolbur group (16SrXII, 'Candidatus Phytoplasma solani')

Molière disease in cherry trees is caused by phytoplasma with the suggested name 'Candidatus Phytoplasma solani' (IRPCM 2004) which belongs to the stolbur group (16SrXII) (Schneider *et al.* 1993; Marcone *et al.* 1999). This phytoplasma was detected in cherry trees growing in northern Italy (Paltrinieri *et al.* 2001, 2008). The leaves of affected trees were curled, smaller, chlorotic and turned red prematurely. Based on Polymerase Chain Reaction/Restriction Fragment Length Polymorphism (PCR/RFLP) analysis, the causal agent was classified to subgroup A of the 16SrXII group. 'Candidatus Phytoplasma australiense' belonging to the same group (16Sr XII-B) was associated with peach yellow leaf roll-like disease in Bolivia (Jones *et al.* 2005). Many of the infected trees were dying or showed symptoms of yellowing of the leaf margin and rolling, drying and necrosis of the leaves, and proliferation of shoots.

Based on the virtual RFLP analysis of 16S rDNA of stolbur phytoplasmas, it was possible to classify them into seven subgroups associated with four 'Candidatus Phytoplasma' species: 'Ca. P. australiense', 'Ca. P. japonicum', 'Ca. P. fragariae', phytoplasma with the suggested name 'Ca. P. solani', and strawberry lethal yellows phy-

toplasma (Quaglino *et al.* 2009). Analysis of the *tuf* gene was also used for molecular characterization of 16SrXII-A phytoplasma (stolbur group) from declining cherry in Italy (Paltrinieri *et al.* 2001).

There are no effective cures for phytoplasmal diseases. Thus, preventative measures such as using healthy plant material, vector control, eliminating those plant hosts which are a source of phytoplasmas etc. are increasingly important in controlling phytoplasmal diseases. Sensitive and reliable methods for detection of phytoplasmas are essential for application of nonchemical measures to control phytoplasmal diseases.

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