ISSN 1392-3196 / e-ISSN 2335-8947
Zemdirbyste-Agriculture, vol. 106, No. 1 (2019), p. 73–80
DOI 10.13080/z-a.2019.106.010

Multilocus sequence analysis of phytoplasmas detected in cherry trees in Poland

Mirosława CIEŚLIŃSKA, Terezia SMOLAREK

Research Institute of Horticulture
Konstytucji 3 Maja 1/3, 96-100 Skierniewice, Poland
E-mail: miroslawa.cieslinska@inhort.pl

Abstract

During 2010–2013 and 2016, shoot samples were collected from symptomatic and asymptomatic 85 sour cherry and 80 sweet cherry trees grown in 16 orchards located in major cherry cultivation areas in Central and Western Poland. Some sour cherry trees showed shoot proliferation, whereas dieback symptoms and leaf roll and yellowing were observed on some sweet cherry trees. The universal phytoplasma-specific primer pairs P1 and P7 derived from the ribosomal sequence within the 16S and 23S rRNA gene sequences and intergenic spacer region were used in direct polymerase chain reaction (PCR) followed by universal primers F1/B6 and primers R16(I)F1/R1 and R16(X)F1/R1 specific for 16SrI and 16SrX groups, respectively. Phytoplasmas were detected in two sweet cherry ('Trzebnica' and 'Kordia II.14') and one sour cherry ('cherry XVI.12') trees.

Restriction fragment length polymorphism (RFLP) analyses conducted after digestion of F1/B6 products (~1.65 kb) with *Hha*I, *Rsa*I, *Ssp*I and *Mse*I enzymes indicated that sweet cherry trees were infected with '*Candidatus* Phytoplasma prunorum' (16SrX-B). The restriction profiles for sour 'cherry XVI.12' sample were indistinguishable from those of the reference strain AY-1 of '*Candidatus* Phytoplasma asteris' (16SrI-B).

Multilocus sequence analysis of 16S DNA plus spacer region, *sec*Y and ribosomal protein (rp) operons confirmed the genetic diversity of phytoplasmas infecting cherry trees and showed the closest relationships 'Trzebnica' and 'Kordia II.14' isolates found in sweet cherry trees to the reference strains of '*Ca*. Ph. prunorum' (16SrX-B). Depending on analysed sequenced region, the phytoplasma infecting sour 'cherry XVI.12' revealed close genetic relationship to phytoplasmas assigned to the different subgroups of aster yellows group (16SrI). *Sec*Y gene was the more informative marker for finer differentiation of the strains of phytoplasmas within 16SrI group and showed the highest genetic similarity of 'cherry XVI.12' isolate with '*Ca.* Ph. asteris'-related strains.

Key words: detection, identification, restriction analysis, phylogeny, sequencing.

Introduction

Phytoplasmas are associated with diseases of several hundreds of plant species in tropical, subtropical, and temperate regions of the world including vegetable and fruit crops and ornamental plants causing losses of yield of rice, potato, maize, corn, cassava, legume, sesame, soybean, grapevine, pome and stone fruits (Lee et al., 2000; Bertaccini et al., 2014). Diseases of stone fruits associated with phytoplasma infection, including apricot chlorotic leaf roll, plum leptonecrosis, peach yellowing and declining of plum, peach and almond, were found to have a common etiology and European stone fruit yellows (ESFY) has been proposed as the common name for phytoplasma-related diseases of European stone fruit trees (Lorenz et al., 1994). Further studies have shown that the disease is associated with '*Candidatus* Phytoplasma prunorum' infection (Seemüller, Schneider, 2004). As '*Ca*. Ph. prunorum' causes economically devastating fruit tree diseases it is included in EPPO A2 List of pests recommended for regulation as quarantine pests (http://www.eppo.int/). '*Ca*. Ph. prunorum' was detected in sweet cherry in the Czech Republic and Poland (Fialová et al., 2004; Cieślińska, Morgaś, 2011) and in sour cherry in Hungary, the Czech Republic and Spain (Varga et al., 2001; Laviña et al., 2004; Ludvikova et al., 2011). Plants of *Prunus* species can be also infected by phytoplasmas trees with few leaf and rosetting symptoms including 16SrI, 16SrII, 16SrIII, 16SrV, 16SrVII, 16SrIX, 16SrX and 16SrXII. The phytoplasmas related to several subgroups of the aster yellows group (16SrI) were associated with stunting, leaf rolling and yellowing of sweet cherry in the Czech Republic (Navràtil et al., 2001), cherry little leaf disease, reduced vigour, leaf drop and die-back of sour cherry in the Czech Republic and in Lithuania (Navràtil et al., 2001; Valiunas et al., 2009 a; Jomantiene et al., 2011). In Iran, sweet cherry trees with few leaf and rosetting symptoms were infected with phytoplasma related to '*Ca.* Ph. aurantifolia' belonging to the peanut witches'-

Please use the following format when citing the article:
Cieślińska M., Smolarek T. 2019. Multilocus sequence analysis of phytoplasmas detected in cherry trees in Poland. Zemdirbyste-Agriculture, 106 (1): 73–80. DOI 10.13080/z-a.2019.106.010

broom group (16SrII) (Zirak et al., 2010). X-disease group phytoplasmas (16SrIII) were associated with the decline of cherry trees in California, USA, in Italy and in Lithuania (Van Steenwyk et al., 1995; Gundersen et al., 1996; Paltrinieri et al., 2001; 2008; Landi et al., 2007; Valiunas et al., 2009 b). '*Candidatus* Ph. ziziphi' belonging to elm yellows group (16SrV) was identified in sour cherry trees showing cherry lethal yellows symptoms (CLY) in China and Italy (Zhu et al., 1998; Paltrinieri et al., 2008), and 'Ca. Ph. fraxini' related to 16SrVII-phytoplasma strains was reported in China in sweet cherry exhibiting fasciation (Li et al., 1997). The occurrence of '*Ca.* Ph. mali' was described in sweet cherry trees in Italy, the Czech Republic and Slovenia (Navràtil et al., 2001; Paltrinieri et al., 2001; Mehle et al., 2007), and '*Ca*. Ph. pyri' (16SrX-C), which is essentially associated with pear decline disease, was detected in sweet cherry in Italy and Poland (Paltrinieri et al., 2001; Cieślińska, Morgaś, 2011). It was also reported that cherry trees growing in Northern Italy were infected with phytoplasmas belonging to stolbur group (16SrXII) (Paltrinieri et al., 2001; 2008).

The aim of this study was to determine the possible association of phytoplasmas with shoot proliferation and dieback of sour cherry and leaf roll and yellowing of the leaves of sweet cherry trees and to examine the variability among phytoplasma strains infecting cherry trees using multilocus sequence typing (MLST).

Materials and methods

During 2010–2013 and 2016, samples consisting of six to ten shoots were collected from symptomatic and asymptomatic 85 sour cherry and 80 sweet cherry trees grown in 16 orchards located in major cherry cultivation areas in Central and Western Poland.

Extraction of nucleic acids and polymerase chain reaction (PCR) analyses. Total DNA was extracted from phloem tissue using the DNeasy Plant Mini Kit (Qiagen, Germany) from fresh phloem tissue of cherry shoots ground in liquid nitrogen. For phytoplasma testing DNA was subjected to PCR with P1/P7 for amplification of a 1800-nucleotide (nt) product of the 16S ribosomal DNA, the spacer region between the 16S and 23S rDNA genes and the 5' of the 23S rDNA of the phytoplasma genome (Table). Nested PCR on P1/P7 amplicons diluted 1:29 in sterile water was then performed using primers F1/B6 that amplify a 1700 nt genome fragment including 16S rRNA and the 16S-23S intergenic spacer region and fragment of 23S rRNA.

The positive samples were used for further analyses to characterize the molecular properties of detected phytoplasma isolates. To confirm phytoplasma identity the P1/P7 amplicons were also re-amplified with primers R16(X)F1/R1, R16(I)F1/R1 produced amplicons of about 1100 nt, specific for 16SrX and 16SrI groups, respectively.

Table. Primer pairs used in polymerase chain reaction (PCR) of '*Candidatus* Phytoplasma prunorum' and '*Ca*. Ph. asteris' from a survey of cherry trees in Central and Western Poland during 2010–2013 and 2016

Genome segment	Approx. size (bp)	Primer pair	References
16S rDNA and 16S-23S	1800	P1/P7[1]	Deng, Hiruki, 1991; Schneider et al., 1995;
	1700	F1/B6[2]	Davis, Lee, 1993; Padovan et al., 1995
16S rDNA	1100	R16(X)F1/R1[2]	Lee et al., 1995;
	1100	R16(I)F1/R1[2]	Lee et al., 1994
L22 and S3 ribosomal protein operon	1200	rp1/rp2[1]	Nakamura et al., 1996;
	750	rp3/rp4[2]	Nakamura et al., 1996;
	1600	rpL2F2/rp(I)R1A1[1]	Martini et al., 2007; Lee et al., 2003;
	1200	rpF1C/rp(I)R1A[2]	Martini et al., 2007; Lee et al., 2003
Protein translocase subunit SecY	2800	L15F1A(I)/MapR1A[1]	Lee et al., 2010;
	1400	AYsecYF1/AYsecYR1[2]	Lee at al., 2006;
	1200	secYF1(X)/secYR1(X)[2]	Lee et al., 2010

[1] – direct primers, [2] – nested primers

For finer molecular characterization of the detected phytoplasmas, ribosomal protein (*rp*122 and *rps*3) and non-ribosomal DNA region secY were also analysed. The *rpl*22 and *rps*3 gene sequences encoding S10 ribosomal protein (rp) operon were amplified in nested PCR using primer pairs rpL2F2/rp(I)R1A1 followed by rpF1C/rp(I)R1A and rp1/rp2 followed by rp3/rp4 specific for '*C*a. Ph. prunorum' and '*C*a. Ph. asteris', respectively. The *sec*Y gene encoding protein translocase subunit SecY were amplified in nested PCR using primer pairs L15F1A(I)/MapR1A followed by secYF1(X)/secYR1(X) specific for '*Ca*. Ph. prunorum' and L15F1A(I)/MapR1A followed by AYsecYF1/AYsecYR1 specific for '*C*a. Ph. asteris'.

All PCR assays were performed with a thermocycler PTC-200 (MJ Research Inc., USA), and 7 µl of the amplification products were separated in 1% agarose gel in 0.5 × Tris-borate-EDTA (TBE) buffer, followed by staining in ethidium bromide and visualization of DNA bands using UV transilluminator (Syngen, USA). The molecular weight of the PCR products was estimated by comparison with 100 bp DNA ladder (Thermo Fisher Scientific, Lithuania). DNA from a sample of asymptomatic cherry trees was included in each PCR assay as negative control, and positive control was the '*Ca*. Ph. prunorum' strain from apricot.

Restriction fragment length polymorphism (RFLP) analyses. Five µl of the PCR products primed with F1/B6 pair were separately digested with by *Hha*I, *Rsa*I, *Ssp*I and *Mse*I enzymes (Thermo Fisher Scientific) to conduct the RFLP analysis of 16S rDNA (16S rRNA gene) and 16S-23S rDNA spacer region. The generated

restriction patterns were analysed by electrophoresis in 8% polyacrylamide gels in 1% TBE buffer and compared with the profiles of the reference strains AY-1 of '*Ca*. Ph. asteris' and AP-15 of '*Ca*. Ph. prunorum' (Bertaccini et al., 2014), and '*Ca*. Ph. prunorum' and '*Ca*. Ph. pyri' from phytoplasma-infected Japanese plum and pear trees, respectively, maintained in collection of the Research Institute of Horticulture in Skierniewice, Poland.

Sequence analysis. The partial sequence of detected phytoplasma strains including 16S rDNA and 16S-23S rDNA spacer region, *rps*3, *rpl*22 and *sec*Y genes were purified from the gels using QIAquick® Gel Extraction Kit (Qiagen) and sequenced in both strains using the same primers employed for amplification. The obtained sequences were aligned using *ClustalW* of the software *DNASTAR's Lasergene* (DNAStar Inc., USA). The consensus sequences were compared with sequences available in GenBank using the BLAST algorithm (http://ncbi.nlm.nih.gov/BLAST/). The genetic relationships of phytoplasma strains and the parallel sequences of the reference strains obtained from GenBank were determined by phylogenetic analysis using the neighbour-joining method with the Tamura 3-parameter model (Tamura et al., 2007) and program *MEGA 5.2*. Bootstrap analysis (1,000 replicates) was performed for statistical significance estimation (values below 70% were collapsed).

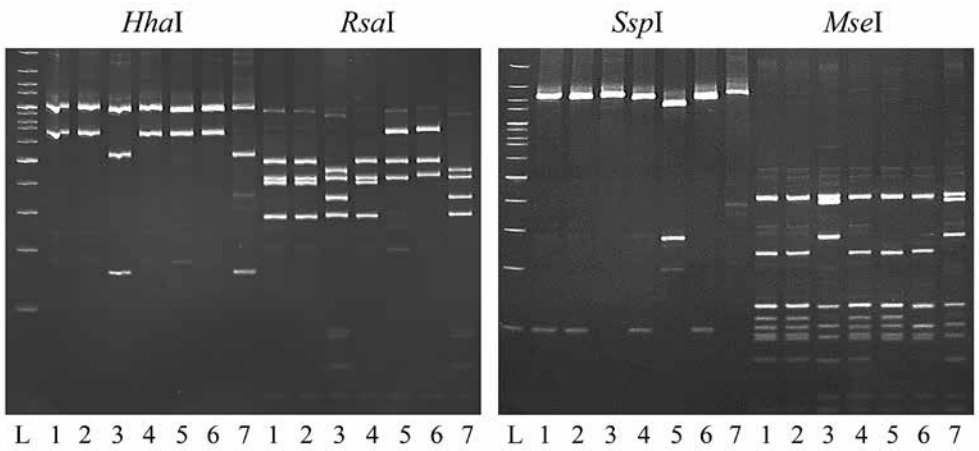
Results and discussion

During the surveys of 16 cherry orchards, 165 leaf samples were collected from asymptomatic and symptomatic cherry trees. Some of sour cherry trees showed shoot proliferation, dieback and off season flowering, whereas on several sweet cherry trees stunting, leaf roll and yellowing were observed.

Direct PCR with the P1/P7 primers did not result in amplification of the expected length products on DNA templates extracted from phloem tissue of shoots collected from surveyed cherry trees. Three samples from symptomatic trees were positive in nested PCR assays with the universal primers F1/B6. Products of the nested PCR with R16(X)F1/R1 specific for apple proliferation (16SrX group) were obtained for 16S rDNA fragments of phytoplasma isolates from two sweet cherry 'Kordia II.14' and 'Trzebnica' trees (data not shown). Stunting, leaf roll and yellowing were observed on these trees. Phytoplasma rDNA fragment from 'cherry XVI.12' was amplified by nested PCR with primer pair R16(I)F1/R1 specific for aster yellows group (16SrI), but not with apple proliferation-specific primers (data not shown).

The PCR products amplified with F1/B6 primers revealed two different profiles after digestion with *Hha*I, *Rsa*I, *Ssp*I and *Mse*I enzymes. Samples 'Kordia II.14' and 'Trzebnica' showed the patterns indistinguishable from those of the European store fruit yellows (ESFY) reference strain of '*Ca*. Ph. prunorum' (16SrX-B) while the 'cherry XVI.12' isolate referable to AY-1 strain of '*Ca*. Ph. asteris' (16SrI-B) (Fig. 1).

The previous study showed that the nucleotide sequence of phytoplasma strains from two sweet cherry trees were identical and shared 99.6–100% similarity with the parallel genome fragment of the '*Ca*. Ph. prunorum' strains: ESFY-G1, ESFY-G2, ESFY-215 and ESFY-2102 (GenBank ID: AY542544, AJ542545, AJ575105 and AM933142, respectively) and that phytoplasma infecting 'cherry XVI.12' formed a monophyletic cluster with the reference strains inside several subgroups of the 16SrI group (Cieślińska, Smolarek, 2015).



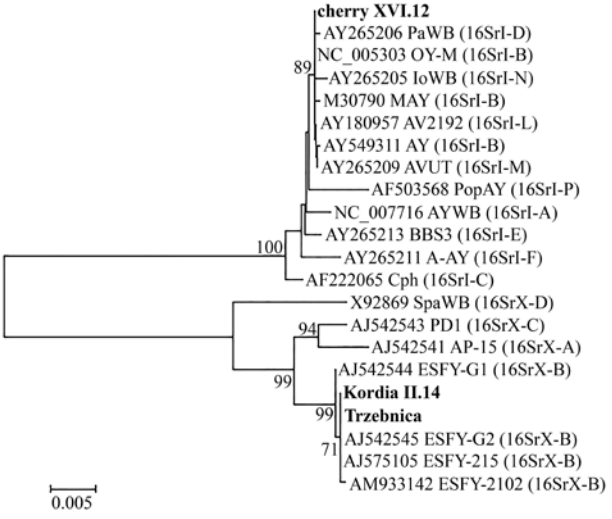
Lanes: L – molecular marker 100 bp, 1 – 'Kordia II.14', 2 – 'Trzebnica', 3 – 'cherry XVI.12', 4 – positive control 16SrX-B, 5 – AP-15 reference strain for 16SrX-A, 6 – positive control 16SrX-C, 7 – AY-1 reference strain for 16SrI-B

Figure 1. Polyacrylamide gel showing the restriction fragment length polymorphism (RFLP) patterns of phytoplasmas ribosomal DNA fragment amplified with F1/B6 primers digested with *Hha*I, *Rsa*I, *Mse*I and *Ssp*I restriction enzymes of cherry samples from a survey of cherry trees in Central and Western Poland during 2010–2013 and 2016

Multiple alignments of 16S rDNA and 16S-23S spacer region revealed that nucleotide sequences of cherry isolates were similar to those obtained previously for 16S rDNA region (Cieślińska, Smolarek, 2015). During the present study it was demonstrated that the sequences of 16S rDNA and 16S-23S spacer region were identical in the both sweet cherry ('Kordia II.14' and 'Trzebnica') samples and in the reference strains of '*Ca*. Ph. prunorum': ESFY-G2, ESFY-215 and ESFY-2102 (GenBank ID: AJ542545, AJ575105 and AM933142, respectively). Substitutions in positions 1607 (A→T) and 1608 (G→A) resulted in 99.7% sequence identity with the reference strain ESFY-G1 (GenBank ID AY542544). Phytoplasma infecting 'cherry XVI.12' formed a cluster

with the reference strains belonging to subgroups B, D, L, M, and N of the 16SrI-B inside the 16SrI group (Fig. 2).

Nucleotide sequences of the 16S rDNA and 16S-23S spacer region of the 'cherry XVI.12', 'Kordia II.14' and 'Trzebnica' isolates were deposited in GenBank under accession numbers LT746085, LT746086 and LT746087, respectively.



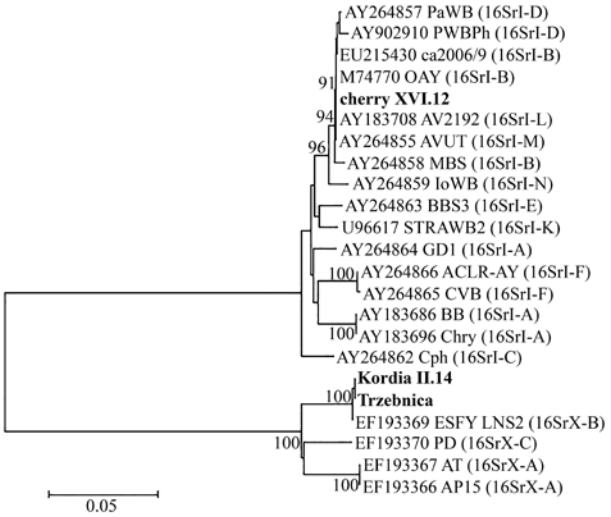
Note. The '*Ca*. Ph. prunorum' and '*Ca*. Ph. asteris' strains identified in this study are indicated in bold; phylogenetic trees were constructed by the neighbour-joining algorithm (MEGA 5.2) and the Kimura 3-parameter nucleotide substitution model in using 1,000 bootstrap replicates; only bootstrap values higher than 70 are shown; the bar indicates the genetic distance as Kimura units.

Figure 2. Phylogenetic analysis of the 16S rDNA and 16S-23S spacer region sequences of phytoplasmas detected from a survey of cherry trees and representative phytoplasma strains from 16SrI and 16SrX group in Central and Western Poland during 2010–2013 and 2016

Although the similarity and phylogenetic analyses of 16S rRNA gene sequence play a pivotal role in the differentiation of groups, they are not effective enough to differentiate some subgroups. Comparisons of phylogenetic analyses of 16S rDNA and 16S-23S spacer region, *rp* and *sec*Y genes proved that they possess greater variability than 16S rDNA hence are more informative markers for finer differentiation of closely related strains (Lee et al., 2006; 2010; Martini et al., 2007; Gao et al., 2008). On the other hand, it was shown that sequences of the ribosomal proteins and *sec*Y gene of the phytoplasmas belonging to the same taxonomic subgroups, but responsible for various diseases were almost identical. One of the examples can be sequences of the *rp* and *sec*Y genes of phytoplasma associated with sweet cherry virescence (subgroup 16SrV-B) in China which were essentially indistinguishable from the analogous sequences of phytoplasma strains involved in jujube witches'-broom (16SrV-B) disease and diseases of many other plants (Wang et al., 2018).

In this study, the 16S-23S rRNA intergenic spacer region, ribosomal protein (*rpl22* and *rps3*) and secY (encodes protein translocase subunit) were selected as supplementary molecular markers for analysis of phytoplasmas detected in cherry trees.

Phylogenetic analysis of *rps*3 and *rpl*22 genes confirmed that the both isolates 'Trzebnica' and 'Kordia II.14' found in sweet cherry trees were related to '*Ca*. Ph. prunorum' (Fig. 3). The sequence of this genome fragment of the both isolates was identical and showed 99.7% similarity with LNS2 strain classified to 16SrX-B subgroup (GenBank ID EF193369). Although the previous study indicated higher variation of *rp* genes than 16S rRNA gene, what implicated its usefulness for differentiation of closely related phytoplasma strains (Martini et al., 2007), sequence analysis of *rps*3 and *rpl*22 genes did not result in differentiation of 'Trzebnica' and 'Kordia II.14' strains of '*Ca*. Ph. prunorum'.



Explanations under Figure 2

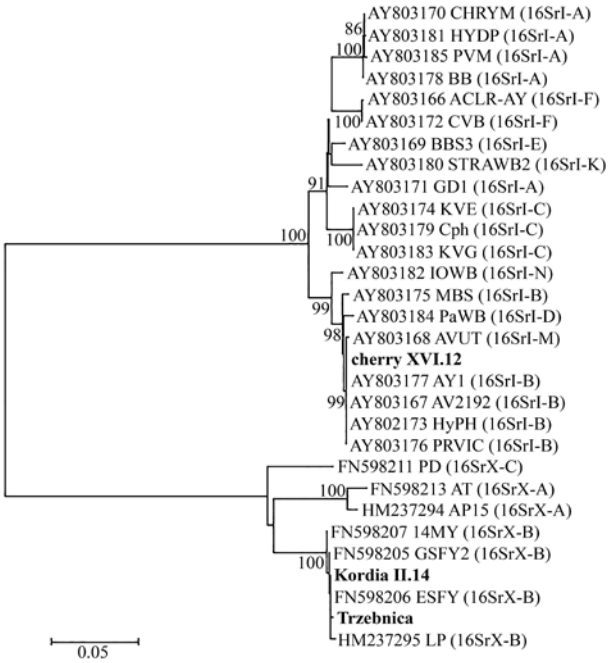
Figure 3. Phylogenetic analysis of the *rps*3 and *rpl*22 genes sequences of phytoplasmas detected from a survey of cherry trees and representative phytoplasma strains from 16SrI and 16SrX groups in Central and Western Poland during 2010–2013 and 2016

Phylogenetic comparison of this genome's fragment of 'cherry XVI.12' isolate with 16 representative strains of phytoplasmas from aster yellows group revealed 96.9–100% (nt) identities of 'cherry XVI.12' isolate with the reference strains of aster yellows phytoplasma. The phytoplasma detected in sour 'cherry XVI.12' showed the highest sequence homology with OAY and ca2006/9 strains of the '*Ca*. Ph. asteris (rpI-B) and the lowest similarity with CVB (96.9%) and ACLR-AY (97%) strains assigned within rpI-F subgroup. Based on these results the phytoplasma detected in sour 'cherry XVI.12' can be enclosed in the rpI-B ribosomal protein subgroup.

Nucleotide sequences of the *rps*3 gene of 'Kordia II.14', 'Trzebnica' and 'cherry XVI.12' isolates were deposited in GenBank under accession numbers MG383522, MG383523 and MG383524, respectively.

Phylogenetic analyses of non-ribosomal genetic loci secY showed high similarity (99.8%) of 'Trzebnica' and 'Kordia II.14' isolates in the partial sequence of this region. The nucleotide substitution (C→T) recognized at the position 491 did not introduce amino acid (AA) change in the secY region as the both amino acid composition (AAC) and acetic acid test (AAT) codons represent asparagine. *Sec*Y gene analysis revealed the identities of 99.7–100% (nt) of the both, 'Trzebnica' and 'Kordia II.14', isolates with the reference strains:

GSFY2, ESFY, 14MY and LP (GenBank ID: FN598205, FN598206, FN598207 and HM237295, respectively) of '*Ca*. Ph. prunorum' (Fig. 4). SecY marker was successfully used in studies of genetic diversity of closely related phytoplasmas within different taxonomic groups. However, it was shown that only three genotypes could be recognized based on sequence analysis of secY region of '*Ca*. Ph. prunorum' isolated from 129 samples, whereas 11 genotypes were separated when *ace*F gene was analysed (Danet et al., 2011).



Explanations under Figure 2

Figure 4. Phylogenetic analysis of the *sec*Y gene sequences of phytoplasmas detected from a survey of cherry trees and representative phytoplasma strains from 16SrI and 16SrX groups in Central and Western Poland during 2010–2013 and 2016

The *sec*Y gene exhibited greater sequence variation than 16S rRNA gene among members of the AY phytoplasma group. Sequence homologies ranged from 94.5% to 100% based on *sec*Y gene sequences of 'cherry XVI.12' isolate and representative strains of the 16SrI subgroups. The nucleotide sequence of the 'cherry XVI.12' isolate showed 100% identity with strains AV2192, HyPh, PRVIC and AY1 classified to 16SrI-B subgroup ('*Ca*. Ph. asteris'). Eight single nucleotide polymorphisms (SNPs) in *sec*Y gene sequence resulted in 99.4% similarity with MBS-another '*Ca*. Ph. asteris'-related strain. The level of similarity 94.5–94.6% was shown between *sec*Y gene sequence of 'cherry XVI.12' isolate and 16SrI-A strains: PVM (71 SNPs), HYPD (68 SNPs), CHRYM (66 SNPs) and ACLR-AY (65 SNPs) as well as CVB strain (66 SNPs) representing subgroup 16SrI-F and strain STRAWB2 (66 SNPs) classified to 16SrI-K subgroup. Sequence analyses of the other two markers resulted in grouping of 'cherry XVI.12' strain in the same clade together with phytoplasmas classified not only to the subgroup 16SrI-B ('*Ca.* Ph. asteris'), but also to the subgroups L and M when analyse *rpl22* and *rps3* genes and D, L, M and N in 16S-23S rRNA intergenic spacer region analysis. Phylogenetic analysis based on secY fragments clearly assigned this isolate to the subgroup B ('*Ca.* Ph. asteris') in aster yellows group. *Sec*Y gene nucleotide sequences of the 'cherry XVI.12', 'Kordia II.14' and 'Trzebnica' strains were deposited in GenBank under accession numbers LT746088, LT746089 and LT746090, respectively.

The results of our study showed that two different phytoplasmas '*Ca*. Ph. prunorum' and '*Ca*. Ph. asteris' were found in cherry trees based on genetic analysis of three genome fragments: 16S rDNA and 16S-23S spacer region, secY and S10 ribosomal protein (rp) operon.

No correlation between symptoms and phytoplasmas presence was noticed as more surveyed trees showed symptoms suggesting phytoplasma infection. It was reported that infection with '*Ca*. Ph. prunorum' does not lead to devastation of sweet and sour cherry orchards, as these trees are latently infected or show only mild symptoms (Kison, Seemüller, 2001). Although this phytoplasma rarely infects cherry, it was identified in Czech Republic in the single sweet cherry tree and manifested stunting, leaf rolling and yellowing. The same symptoms were observed on sweet cherries 'Kordia II.14' and 'Trzebnica'.

In turn, a decline of sweet cherry trees in Italy was associated with European stone fruit yellows disease caused by '*Ca*. Ph. prunorum' (Paltrinieri et al., 2001; Landi et al., 2007). Stunting, chlorotic leaf roll, short internodes, wilting and dieback of branches were observed on several cherry trees infected with '*Ca*. Ph. prunorum' in Poland; however, some of the positively tested cherries became symptomless (Cieślińska, Morgaś, 2011). '*Ca*. Ph. prunorum' was also detected in sour cherry trees surveyed in East Bohemia, the Czech Republic showing leaf roll and yellowing symptoms, sparse foliage, smaller size of leaves and fruits (Ludvikowa et al., 2011).

Although aster yellows group is one of the most diverse phytoplasma groups associated with diseases of many plant species, including economically important ones (Lee et al., 2004), it is mainly associated with diseases of perennial plants. However, their host range is broader and includes cherry and other fruit tree species.

Navràtil et al. (2001) reported that in the Czech Republic sour cherry trees with small leaves, reduced vigour, and die-back were mainly infected not only by '*Ca*. Ph. prunorum', but also by '*Ca*. Ph. asteris'. Phytoplasmas from 16SrI-B subgroup were also reported in sour cherry in Lithuania (Valiūnas et al., 2007) and mahaleb in Hungary (Varga et al., 2001). The further study proved that sour cherry could be affected by phytoplasmas assigned to different subgroups within aster yellows group. It was found that mild yellowing of leaves, bunchy little leaf growths and leaf drop observed on sour cherry in Lithuania were associated with phytoplasma representing two distinct lineages related to clover phyllody phytoplasma and classified to a subgroup 16SrI-(R/S)C (formerly 16SrI-C) strain exhibiting rRNA interoperon sequence heterogeneity (Jomantiene et al., 2011). Cherry little leaf (ChLL) disease exhibiting shoot proliferation, small leaves and a decline of sour cherry trees in Lithuania was associated with phytoplasma classified to a new subgroup – 16SrI-Q (Valiunas et al., 2009 a). Although the similar symptoms was also observed during presented study, the sequence analysis of genome fragments revealed that phytoplasma found in sour 'cherry XVI.12' was closely related to '*Ca*. Ph. asteris'.

The low incidence of the phytoplasma diseases of cherry suggests that large scale exchange of nursery material could be involved in the appearance of epidemic strains in cherry production areas. The other possibility is transmission of the phytoplasmas by insect vectors. The individuals of *Cacopsylla pruni* were reported to be infected with '*Ca*. Ph. prunorum'; however, their role in phytoplasma transmission seems to be marginal (Warabieda et al., 2018). In turn, aster yellows phytoplasmas, including '*Ca.* Ph. asteris', have wide host and vector ranges (Lee et al., 2004) and might be transmitted by leafhopper from other crops.

Conclusions

1. Only three among 165 tested cherry trees revealed to be infected with phytoplasmas. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis demonstrated the presence of phytoplasma assigned to 16SrX-B group ('*Candidatus* Phytoplasma prunorum') in sweet cherry 'Kordia II.14' and 'Trzebnica' trees, while positively-tested sour 'cherry XVI.12' tree was infected by phytoplasma belonging to 16SrI-B ('*Ca*. Ph. asteris').

2. Phylogenetic analysis based on the ribosomal sequence within the 16S and 23S rRNA genes and intergenic spacer region, *sec*Y gene as well as *rps*3 and *rpl22* genes, confirmed that isolates 'Kordia II.14' and 'Trzebnica' were related to '*Ca.* Ph. prunorum' strains, whereas 'cherry XVI.12' isolate was closest in relation to '*Ca.* Ph. asteris' strains.

3. The *sec*Y gene exhibited greater sequence variation than 16S rRNA gene plus spacer region and ribosomal protein (rp) *rps*3 and *rpl*22 genes among '*Ca.* Ph. asteris'-related strains within aster yellows phytoplasma (16SrI) group and allowed us to clearly assign 'cherry XVI.12' isolate to the subgroup B.

4. This is the first report of multilocus sequence analysis of phytoplasmas found in cherry trees in Poland.

Acknowledgements

The study was conducted in the frame of COST Action FA1104 and financially supported by National Science Center in Poland (project UMO-2013/08/M/NZ9/00138).

Received 13 12 2017
Accepted 21 03 2018

References

1. Bertaccini A., Duduk B., Paltrinieri S., Contaldo N. 2014. Phytoplasmas and phytoplasma diseases: a severe threat to agriculture. American Journal of Plant Science, 5: 1763–1788. https://doi.org/10.4236/ajps.2014.512191
2. Cieślińska M., Morgaś H. 2011. Detection and identification of '*Candidatus* Phytoplasma prunorum', '*Candidatus* Phytoplasma mali' and '*Candidatus* Phytoplasma pyri' in stone fruit trees in Poland. Journal of Phytopathology, 159 (4): 217–222. https://doi.org/10.1111/j.1439-0434.2010.01752.x
3. Cieślińska M., Smolarek T. 2015. Molecular diversity of phytoplasmas infecting cherry trees in Poland. Phytopathogenic Mollicutes, 5 (suppl. 1): S31–S32. https://doi.org/10.5958/2249-4677.2015.00013.4
4. Danet J. L., Balakishiyeva G., Cimerman A., Sauvion N., Marie-Jeanne V., Labonne G., Laviña A., Batlle A., Križanac I., Škorić D., Ermacora P., Ulubaş Serçe Ç., Çağlayan K., Jarausch W., Foissac X. 2011.Multilocus sequence analysis reveals the genetic diversity of European fruit tree phytoplasmas and the existence of interspecies recombination. Microbiology, 157 (2): 438–450. https://doi.org/10.1099/mic.0.043547-0
5. Davis R. E., Lee I.-M. 1993. Cluster-specific polymerase chain reaction amplification of 16S rDNA sequences for detection and identification of mycoplasma-like organisms. Phytopathology, 83: 1008–1011. https://doi.org/10.1094/Phyto-83-1008
6. Deng S., Hiruki C. 1991. Genetic relatedness between two nonculturable mycoplasmalike organisms revealed by nucleic acid hybridization and polymerase chain reaction. Phytopathology, 81 (12): 1475–1479. https://doi.org/10.1094/Phyto-81-1475
7. Fialová R., Navrátil M., Válová P., Lauterer P., Kocourek F., Poncarová-Voráčková Z. 2004. Epidemiology of European stone fruit yellows phytoplasma in the Czech Republic. Acta Horticulturae, 657: 483–487. https://doi.org/10.17660/ActaHortic.2004.657.78
8. Gao R., Zhang G. M., Lan Y. F., Zhu T. S., Yu X. Q., Zhu X. P., Li X. D. 2008. Molecular characterization of phytoplasma associated with rose witches'-broom in China. Journal of Phytopathology, 156: 93–98. https://doi.org/10.1111/j.1439-0434.2007.01322.x
9. Gundersen D. E., Lee I.-M., Schaff D. A., Harrison N. A., Chang C. J., Davis R. E., Kingsbury D. T. 1996. Genomic diversity and differentiation among phytoplasma strains in 16S rRNA groups I (aster yellows and related phytoplasmas) and III (X disease and related phytoplasmas). International Journal of Systematic Bacteriology, 46: 64–75. https://doi.org/10.1099/00207713-46-1-64
10. Jomantiene R., Zhao Z., Lee I.-M., Davis R. E. 2011. Phytoplasmas infecting sour cherry and lilac represent two distinct lineages having close evolutionary affinities with clover phyllody phytoplasma. European Journal of Plant Pathology, 130 (1): 97–107. https://doi.org/10.1007/s10658-010-9735-5
11. Kison H., Seemüller E. 2001. Differences in strain virulence of the European stone fruit yellows phytoplasma and susceptibility of stone fruit trees on various rootstocks to this pathogen. Journal of Phytopathology, 149 (10): 533–541. https://doi.org/10.1046/j.1439-0434.2001.00671.x
12. Landi F., Prandini A., Paltrinieri S., Mori N., Bertaccini A. 2007. Detection of different types of phytoplasmas in stone fruit orchards in northern Italy. Bulletin of Insectology, 60 (2): 163–164.
13. Laviña A., Sabaté J., García-Chapa M., Batle A., Torres E. 2004. Occurrence and epidemiology of European stone fruit yellows phytoplasma in Spain. Acta Horticulturae, 657: 489–494. https://doi.org/10.17660/ActaHortic.2004.657.79
14. Lee I.-M., Gundersen D. E., Hammond R. W., Davis R. E. 1994. Use of mycoplasmalike organisms (MLOs) group specific oligonucleotide primers for nested-PCR assays to detect mixed-MLO infections in a single host plant. Phytopathology, 84: 559–566. https://doi.org/10.1094/Phyto-84-559
15. Lee I.-M., Bertaccini A., Vibio M., Gundersen D. E. 1995. Detection of multiply phytoplasmas in perennial fruit trees with decline symptoms in Italy. Phytopathology, 85: 728–735. https://doi.org/10.1094/Phyto-85-728

16. Lee I.-M., Davis R. E., Gundersen-Rindal D. E. 2000. Phytoplasma: phytopathogenic mollicutes. Annual Review of Microbiology, 54: 221–255. https://doi.org/10.1146/annurev.micro.54.1.221
17. Lee I.-M., Martini M., Bottner K. D., Dane R. A., Black M. C., Troxclair N. 2003. Ecological implications from a molecular analysis of phytoplasmas involved in an aster yellows epidemic in various crops in Texas. Phytopathology, 93: 1368–1377. https://doi.org/10.1094/PHYTO.2003.93.11.1368
18. Lee I.-M., Gundersen-Rindal D. E., Davis R. E., Bottner K. D., Marcone C., Seemüller E. 2004. '*Candidatus* Phytoplasma asteris', a novel phytoplasma taxon associated with aster yellows and related diseases. International Journal of Systematic and Evolutionary Bacteriology, 54 (4): 1037–1048. https://doi.org/10.1099/ijs.0.02843-0
19. Lee I.-M., Zhao Y., Bottner K. D. 2006. *Sec*Y gene sequence analysis for finer differentiation of diverse strains in the aster yellows phytoplasma group. Molecular and Cellular Probes, 20: 87–91. https://doi.org/10.1016/j.mcp.2005.10.001
20. Lee I.-M., Bottner-Parker K., Zhao Y., Davis R., Harrison N. 2010. Phylogenetic analysis and delineation of phytoplasmas based on *sec*Y gene sequences. International Journal of Systematic and Evolutionary Microbiology, 60 (12): 2887–2897. https://doi.org/10.1099/ijs.0.019695-0
21. Li H. H., Qiu B. S., Shi C. L., Jin K. X., Zhou Q., Huang X. J. 1997. PCR amplification of 16S rDNA of phytoplasma associated with cherry fasciated disease and RFLP analysis. Forest Research, 10: 478–481.
22. Lorenz K.-H., Dosba F., Poggi-Pollini C., Llácer G., Seemüller E. 1994. Phytoplasma diseases of Prunus species in Europe are caused by genetically similar organisms. Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz, 101 (6): 567–575.
23. Ludvikova H., Franova J., Sucha J. 2011. Phytoplasmas in apricot, peach and sour cherry orchards in East Bohemia, Czech Republic. Bulletin of Insectology, 64 (suppl.): S67–S68.
24. Martini M., Lee I.-M., Bottner K. D., Zhao Y., Botti S., Bertaccini A., Harrison N. A., Carraro L., Marcone C., Khan A. J., Osler R. 2007. Ribosomal protein gene-based phylogeny for finer differentiation and classification of phytoplasmas. International Journal of Systematic and Evolutionary Microbiology, 57: 2037–2051. https://doi.org/10.1099/ijs.0.65013-0
25. Mehle N., Brzin J., Boben J., Hren M., Frank J., Petrovič N., Gruden K., Dreo T., Žežlina I., Seljak G., Ravnikar M. 2007. First report of '*Candidatus* Phytoplasma mali' in *Prunus avium*, *P. armeniaca* and *P. domestica*. Plant Pathology, 56: 721. https://doi.org/10.1111/j.1365-3059.2007.01597.x
26. Nakamura H., Yoshikawa N., Takahashi T., Sahashi N., Kubono T., Shoji T. 1996. Evaluation of primer pairs for the reliable diagnosis of paulownia witches'-broom disease using a polymerase chain reaction. Plant Disease, 80 (3): 302–305. https://doi.org/10.1094/PD-80-0302
27. Navràtil M., Válová P., Fialová R., Petrová K., Fránová J., Nebesářová J., Poncarová-Voráčková Z., Karešová R. 2001. Survey for stone fruit phytoplasmas in Czech Republic. Acta Horticulturae, 550: 377–382. https://doi.org/10.17660/ActaHortic.2001.550.56
28. Padovan A. C., Gibb K. S., Bertaccini A., Vibio M., Bonfiglioli R. E., Magarey P. A. Sears B. B. 1995. Molecular detection of the Australian grapevine yellows phytoplasma and comparison with a grapevine yellows phytoplasma from Emilia-Romagna in Italy. Australian Journal of Grape and Wine Research, 1: 25–31. https://doi.org/10.1111/j.1755-0238.1995.tb00074.x
29. Paltrinieri S., Martini M., Stefani E., Pondrelli M., Fideghelli C., Bertaccini A. 2001. Phytoplasma infection in peach and cherry in Italy. Acta Horticulturae, 550: 365–370. https://doi.org/10.17660/ActaHortic.2001.550.54
30. Paltrinieri S., Bertaccini A., Lugaresi C. 2008. Phytoplasmas in declining cherry plants. Acta Horticulturae, 781: 409–415. https://doi.org/10.17660/ActaHortic.2008.781.58
31. Schneider B., Seemüller E., Smart C. D., Kirkpatrick B. C. 1995. Phylogenetic classification of plant pathogenic mycoplasma-like organisms or phytoplasmas. Razin S., Tully J. G. (eds). Molecular and diagnostic procedures in mycoplasmology, vol. 1, p. 369–380. https://doi.org/10.1016/B978-012583805-4/50040-6
32. Seemüller E., Schneider B. 2004. Taxonomic description of '*Candidatus* Phytoplasma mali' sp. nov., '*Candidatus* Phytoplasma pyri' sp. nov., '*Candidatus* Phytoplasma prunorum' sp. nov., the causal agents of apple proliferation, pear decline and European stone fruit yellows, respectively. International Journal of Systematic and Evolutionary Microbiology, 54 (4):1217–1226. https://doi.org/10.1099/ijs.0.02823-0
33. Tamura K., Dudley J., Nei M., Kumar S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution, 24 (8): 1596–1599. https://doi.org/10.1093/molbev/msm092
34. Valiūnas D., Jomantienė R. Davis R. E. 2007. Phytoplasmas detected in cultivated fruit plants in Lithuania. Bulletin of Insectology, 60 (2): 139–140.
35. Valiunas D., Jomantiene R., Davis R. E. 2009 (a). Establishment of a new phytoplasma subgroup, 16SrI-Q, to accommodate a previously undescribed phytoplasma found in diseased cherry in Lithuania. Journal of Plant Pathology, 91 (1): 71–75.
36. Valiunas D., Jomantiene R., Ivanauskas A, Abraitis R., Staniene G., Zhao Y., Davis R. E. 2009 (b). First report of a new phytoplasma subgroup, 16SrIII-T, associated with decline disease affecting sweet and sour cherry trees in Lithuania. Plant Disease, 93 (3): 550. https://doi.org/10.1094/PDIS-93-5-0550B
37. Van Steenwyk R. A., Kirkpatrick B. C., Purcell A. H., Fouche C. F., Grant J. A., Uyemoto J. K., 1995. Evaluation of an abatement program for western X-disease in sweet cherry. Plant Disease, 79 (10): 1025–1028. https://doi.org/10.1094/PD-79-1025
38. Varga K., Kőlber M., Ember I., Erdős Z., Biró E., Paltrinieri S., Martini M.., Bertaccini A. 2001. Identification of phytoplasmas infecting sour cherry in Hungary. Acta Horticulturae, 550: 383–388. https://doi.org/10.17660/ActaHortic.2001.550.57
39. Wang J., Liu Q., Wei W., Davis R. E., Tan Y., Lee I.-M., Zhu D., Wei H., Zhao Y. 2018. Multilocus genotyping identifies a highly homogeneous phytoplasma lineage associated with sweet cherry virescence disease in China and its carriage by an erythroneurine leafhopper. Crop Protection, 106: 13–22. https://doi.org/10.1016/j.cropro.2017.12.005
40. Warabieda W., Soika G., Cieślińska M. 2018. The occurrence of *Cacopsylla pruni* on *Prunus* species in Poland and its significance as a vector of '*Candidatus* Phytoplasma prunorum'. Zemdirbyste-Agriculture, 105 (2): 177–182. https://doi.org/10.13080/z-a.2018.105.023
41. Zhu S. F., Hadidi A., Gundersen D. E., Lee I.-M., Zhang C. L. 1998. Characterization of the phytoplasmas associated with cherry lethal yellows and jujube witches'-broom in China. Acta Horticulturae, 472: 701–714. https://doi.org/10.17660/ActaHortic.1998.472.94
42. Zirak L., Bahar M., Ahoonmanesh A. 2010. Characterization of phytoplasmas related to '*Candidatus* Phytoplasma asteris' and peanut WB group associated with sweet cherry diseases in Iran. Journal of Phytopathology, 158 (6): 63–65. https://doi.org/10.1111/j.1439-0434.2009.01566.x

ISSN 1392-3196 / e-ISSN 2335-8947
Zemdirbyste-Agriculture, vol. 106, No. 1 (2019), p. 73–80
DOI 10.13080/z-a.2019.106.010

Fitoplazmų, nustatytų vyšnių ir trešnių medžiuose Lenkijoje, daugialokusinės sekos analizė

M. Cieślińska, T. Smolarek

Skiernevicų sodininkystės tyrimų institutas, Lenkija

Santrauka

2010–2013 ir 2016 metais buvo surinkti ūgliai iš simptomatinių ir asimptomatinių 85 vyšnių bei 80 trešnių medžių, augusių 16-oje sodų pagrindiniuose jų auginimo regionuose Centrinėje ir Vakarų Lenkijoje. Kai kurie vyšnių medžiai demonstravo ūglių proliferaciją, o ant kai kurių trešnių medžių buvo pastebėtos lapų džiūsna, lapų garbanė ir gelta. Universalios fitoplazmai specifinės pradmenų poros P1 ir P7 susintetintos pagal ribosomų 16S bei 23S rRNA genų ir nekoduojančio tarpgeninio regiono sekas buvo naudojamos taikant tiesioginęe polimerazės grandininę reakciją (PGR). Po to naudoti universalūs pradmenys F1/B6 ir pradmenys, specifiniai 16SrI ir 16SrX grupėms R16(I)F1/R1 ir R16(X)F1/R1. Phitoplazmos buvo nustatytos dviejuose trešnių ‘Trzebnica’ bei ‘Kordia II.14’ ir viename vyšnių ‘XVI.12’ medžiuose.

Restrikcinių fragmentų ilgio polimorfizmo (RFLP) analizės rezultatai, gauti suskaidžius restrikcijos endonukleazėmis *Hha*I, *Rsa*I, *Ssp*I ir *Mse*I pradmenų poros F1/B6 PGR reakcijos produktus (~1,65 kb), parodė, kad mėginyje trešnių medžiai buvo užsikrėtę ‘*Candidatus* Phytoplasma prunorum’ (16SrX-B), o vyšnių ‘XVI.12’ profiliai nesiskyrė nuo kontrolinės padermės AY-1 ‘*Candidatus* Phytoplasma asteris’ (16SrI-B).

16S geno, nekoduojančio tarpgeninio regiono, *sec*Y ir rp operono sekostaita patvirtino fitoplazmų, infekuojančių vyšnių medžius, genetinę įvairovę ir parodė artimą ‘Trzebnica’ bei ‘Kordia II.14’ izoliatų ryšį su kontrolinėmis padermėmis ‘*Ca*. Ph. prunorum’ (16SrX-B). Vyšnią ‘XVI.12’ infekuojanti fitoplazma atskleidė artimą genetinį ryšį su fitoplazmomis, priskiriamoms skirtingiems fitoplazminės ligos (angl. *aster yellows*) grupės (16SrI) pogrupiams. *Sec*Y genas buvo informatyvesnis žymeklis smulkesniam fitoplazmų padermių diferencijavimui 16SrI grupėje ir turėjo didžiausią genetinį ‘XVI.12’ izoliato panašumą su padermėmis, susijusiomis su ‘*Ca.* Ph. asteris’.

Reikšminiai žodžiai: filogenija, identifikavimas, nustatymas, restrikcinė analizė, sekoskaita.