Journal of Plant Diseases and Protection, **117** (5), 206–213, 2010, ISSN 1861-3829.

Diversity of Phytoplasmas Infecting Fruit Trees and Their Vectors in Croatia

Diversität von Obstbaum infizierenden Phytoplasmen und ihren Vektoren in Kroatien

I. Križanac[1], I. Mikec[†,1], Ž. Budinščak[1], M. Šeruga Musić[2] & D. Škorić[2,*]
[1] Institute for Plant Protection, HCPHS, Svetošimunska 25, Zagreb, Croatia
[2] Department of Biology, Faculty of Science, University of Zagreb, Croatia
* Corresponding author, e-mail dijana@botanic.hr

Received 16 March 2010, accepted 27 July 2010

Abstract

A survey started in 2002 (Križanac et al. 2008) was continued to determine the diversity of phytoplasmas infecting fruit trees and their vectors in Croatia. Leaf and twig samples from 32 commercial orchards located in all major fruit growing regions of Croatia, were visually inspected for symptoms and tested for the presence of the phytoplasma 16S rDNA as were adjacent symptomless wild *Prunus* species, and potential insect vectors from seven orchards. Phytoplasmas belonging to '*Candidatus* Phytoplasma pyri' and '*Ca*. P. prunorum' (ribosomal subgroups 16SrX-C and 16SrX-B, respectively) were the most widespread and had the highest incidence in pears and stone fruits, respectively. '*Ca*. P. asteris' (16SrI-B) was found sporadically in both fruit trees and vectors, while phytoplasmas of the stolbur group (16SrXII-A) was frequently found in pear. Mixed infections were detected in one pear and one Japanese plum. The high incidence and wide geographical distribution of stolbur phytoplasma in pears along with its presence in *Cacopsyla pyri*, the main vector of pear phytoplasma, calls for investigation of the role of this phytoplasma in the development of pear decline in Croatia. *Cacopsylla pyrisuga*, a suspected '*Ca*. P. pyri' psyllid vector, was also found to harbour stolbur phytoplasma. This study revealed higher phytoplasma diversity in fruit trees and vectors than anticipated.

Key words: 16S rDNA PCR-RFLP, aster yellows, *Cacopsylla pyri*, *C. pyrisuga*, *C. pruni*, '*Candidatus* Phytoplasma pyri', '*Ca*. P. prunorum', stolbur

Zusammenfassung

In Kroatien wurde, in Fortsetzung eines 2002 begonnenen Projektes (Križanac et al. 2008), die Diversität Obstbaum infizierender Phytoplasmen und ihrer Vektoren untersucht. Blätter und Zweige von insgesamt 32 kommerziell geführten Obstanlagen aus allen Obstbaugebieten des Landes wurden im Sommer und Herbst visuell hinsichtlich ihrer Symptome untersucht. Ebenfalls wurde das Material – ebenso wie Proben von benachbarten, symptomlosen wilden Prunus-Arten und Insekten aus sieben Obstanlagen – auf das Vorkommen von 16 rDNA getestet. Phytoplasmen, die den Untergruppen 16SrX-C und 16SrX-B ('*Candidatus* Phytoplasma pyri' und '*Ca*. P. prunorum') angehören, waren am weitesten verbreitet mit dem höchsten Befall an Birnenbäumen und Steinfrüchten. '*Ca*. P. asteris' (16SrI-B) wurde sporadisch sowohl in Obstbäumen als auch in den Vektoren gefunden, während Phytoplasmen der Stolbur-Gruppe (16SrXII-A) nur gelegentlich in Birnen nachgewiesen werden konnten. Mischinfektionen wurden einmal in *Pyrus communis* und einmal in einer japanischen Prunus-Art nachgewiesen. Hoher Befall und weite geographische Verbreitung von Stolbur-Phytoplasmen (16SrXII-A) konnte in Birnen und dem Hauptvektor *Cacopsyla pyri* gezeigt werden. Außerdem konnte *Cacopsylla pyrisuga* als Vektor für Stolbur-Phytoplasmen nachgewiesen werden. Die Untersuchungen ergaben insgesamt eine höhere Diversität an Phytoplasmen in Obstbäumen als erwartet wurde.

Stichwörter: 16S rDNA PCR-RFLP, aster yellows, *Cacopsylla pyri*, *C. pyrisuga*, *C. pruni*, '*Candidatus* Phytoplasma pyri', '*Ca*. P. prunorum', Stolbur

1 Introduction

Phytoplasmas are non-cultivable, wall-less prokaryotes belonging to the monophyletic clade of the class *Mollicutes* (IRPCM 2004). They inhabit phloem tissue of plants, as well as various cell types of insects and their life cycle involves replication in both plant and insect hosts (Hogenhout et al. 2008). These endocellular parasites are associated with economically important diseases in several hundred plant species (Bertaccini 2007), which is reflected in their historical descriptive names. Due to the inability of growing phytoplasmas in axenic cultures, their phylogeny is mainly based on conserved genes sequence analyses, particularly on the 16S rRNA gene (Gundersen et al. 1994; IRPCM 2004), a common bacterial phylogenetic marker. Apple proliferation (AP), European stone fruit yellows (ESFY) and pear decline (PD), phytoplasmas associated with devastating diseases of fruit trees, are classified as subgroups A, B and C, of the ribosomal group 16SrX, respectively (Lee et al. 2000). AP, PD and ESFY phytoplasmas were assigned to different taxa on the basis of differences in their host specificity, vector transmission and phylogenetic markers different from the 16S rRNA gene (IRPCM 2004; Seemüller and Schneider 2004), namely '*Candidatus* Phytoplasma mali', '*Ca*. P. pyri' and '*Ca*. P. prunorum', respectively. The chromosomes of all three phytoplasmas appear to be linear, an unusual feature for phytoplasmas (Kube et al. 2008).

The three above-mentioned phytoplasmas, AP, PD and ESFY, can cause economically important losses in fruit trees by significantly reducing tree vigour and yield. They are vectored in nature by psyllids. '*Ca*. P. pyri' is transmitted by *Cacopsylla pyricola* and *C. pyri* (Jensen et al. 1964; Carraro et al. 1998a); '*Ca*. P. mali' by *C. costalis* (syn. *C. picta*) and *C. melanoneura* (Frisinghelli et al. 2000; Tedeschi et al. 2003; Jarausch et al. 2003), and '*Ca*. P. prunorum' by *C. pruni* (Carraro et al. 1998b). It is suspected that '*Ca*. P. pyri' can also be transmitted by *C. pyrisuga* (Kučerova et al. 2007). All vectors are present in Europe.

The occurrence of pear decline and apple proliferation diseases in apple and pear trees was first reported in Croatia in the nineteen hundred seventies and eighties (Cvjetković 1976; Šarić and Cvjetković 1985; Cvjetković et al. 1987), respectively. Williams pear trees grafted on pear seedling rootstocks from Dalmatia (coastal part of Croatia) were severely affected at the time. Besides typical leaf reddening and downward curling, midvein necrosis and general decline symptoms, discoloured necrotic line at the graft union were regularly recorded (pers. obs.). The proliferation symptoms were

the most prominent in Red Delicious apples of Ormož (Slovenia) and north-western Croatia, however, without clear indication of the Croatian locations. Over 70% of apple trees in those areas had proliferation symptoms. Their fruits were very small and insipid. The presence of phytoplasma cells in leaf mid vein sieve tubes of apples and pears was demonstrated by electron microscopy. Oxytetracycline therapy of pear trees over a period of two years resulted in recovery (Šarić and Cvjetković 1985).

In spite of the economic importance of fruit tree phytoplasmas in Croatia, a systematic survey of these pathogens and their potential insect vectors is lacking. In the last decade, PD symptoms have been frequently observed, especially in newly planted pear orchards of continental Croatia. In 2002, the first monitoring of symptoms of phytoplasma in pears was started. The detection of phytoplasma in pears, apples, as well as in their vectors was done in 2003 and 2004 (Križanac et al. 2008). The aim of the current research was to extend the survey by including observations from more locations and years, and by including more fruit types, namely stone fruits in addition to pome, as well as their potential psyllid vectors.

2 Materials and methods

2.1 Materials

Collection of plant and insect samples. Thirty-two commercial orchards, which grew either pome or stone fruits, and were located in one of the three fruit growing regions in Croatia (Fig. 1) were chosen for the survey. The separation in regions is based on agro-climatical zoning (Fig. 1). The regions also differ in the way orchards are managed. They will be referred to as continental west (CW), continental east (CE) and Adriatic (AD). The orchards were visually inspected for the presence of AP-, PD- and ESFY-like symptoms during summer and autumn in four consecutive years (2002–2005). Leaf and twig samples were collected from symptomatic fruit trees and sporadically from adjacent asymptomatic wild *Prunus* species and transferred to $CaCl_2$ to dry. In 2004 and 2005, potential insect vectors were monitored and collected using the beating method (Steiner 1967) from seven orchards distributed throughout the three regions. In addition, in 2006 and 2007, psyllids were monitored in ten pear and three apple orchards located in the CW and CE regions. Five to fifteen psyllids were captured per location and kept in 95% ethanol until species identification and nucleic acids extraction.

Phytoplasma reference strains. The phytoplasma reference strains were obtained from the collection of DiSTA Phytoplasmology laboratory, University of Bologna, Italy (IRPCM 2004; http://137.204.42.130/person/collection-september_2003.pdf) either as nucleic acids extracts or micropropagated plants of *Catharanthus roseus* L. Don, the main experimental phytoplasma plant host. The following reference strains were used in PCR and RFLP analysis for comparison: AP-15 (ribosomal subgroup 16SrX-A); GSFY-1, GSFY-2 (16SrX-B); PD (16SrX-C); SA-1, STOL (16SrXII-A) and HYDB (16SrI-B).

2.2 Methods

Nucleic acids extraction. Total nucleic acid (TNA) was extracted from plant samples following the procedure described by Angelini et al. (2001) with modifications introduced by Šeruga et al. (2003). Approximately 200 mg of $CaCl_2$-dried leaf midribs and/or phloem tissue scrapings was used. After insect species identification, TNA was extracted from batches of five insects per species. Per location, at least one batch of insects per species was analyzed for phytoplasma presence.

DNA amplification. Direct PCR assays were performed using phytoplasma universal primers R16F1/R0 (Lee et al. 1995) and first nested PCR was primed by R16F2n/R2 (Gundersen and Lee 1996). Nested PCRs, using group specific primers R16(X)F1/R1 and R16(I)F1/R1 on R16F2n/R2 amplicons as templates, were performed (Lee et al. 1994) for the confirmation of phytoplasma identity in mixed infections as well as for obtaining more sensitive diagnosis. For all other experimental conditions and procedures see Šeruga et al. (2003).

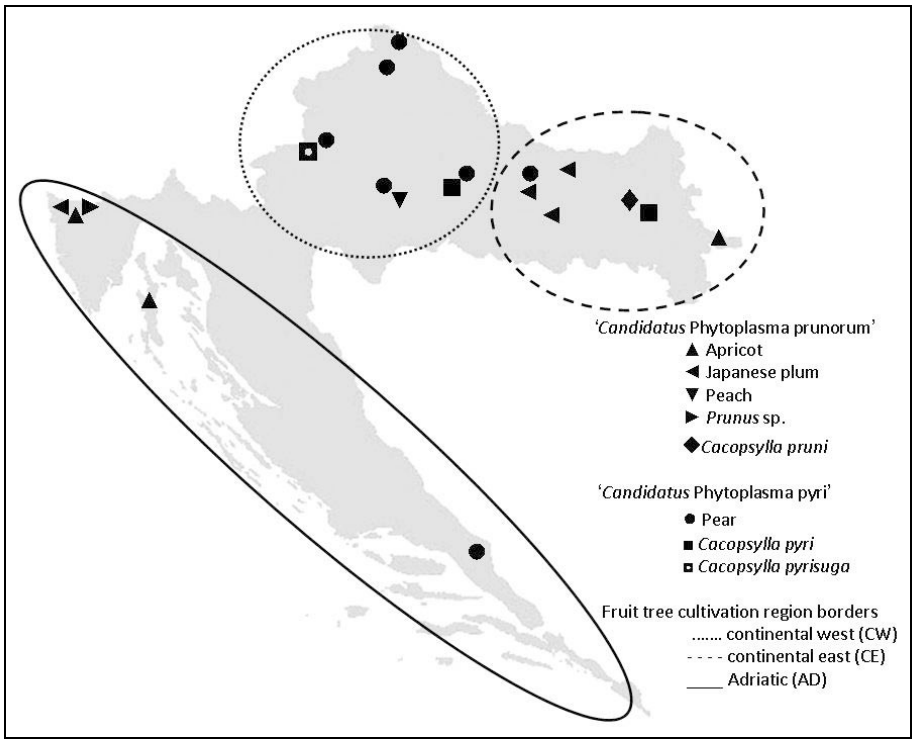

Fig. 1: Geographical distribution of fruit trees and psyllids infected by phytoplasma of ribosomal group 16SrX in Croatia.

RFLP analysis. R16F2n/R2 and R16(I)F1/R1 amplicons were digested with *Tru*9I (= *Mse*I) restriction endonuclease (Roche Diagnostics GmbH, Mannheim, Germany). For samples showing profiles of the 16SrX group, additional digestions of R16F2n/R2 amplicons with *Rsa*I and *Ssp*I (Fermentas, Vilnius, Lithuania) were performed in order to determine the ribosomal subgroup affiliation. Obtained fragments were separated by electrophoresis through 5% polyacrylamide gel and stained with ethidium-bromide. RFLP patterns were compared with those of reference strains.

3 Results

3.1 Symptoms

The most prominent symptoms of all monitored fruit tree species were observed in pear orchards, especially those in the CW region (Fig. 1). In the majority of the sampled pear trees, symptoms reminiscent of PD were observed, encompassing slow decline with leaf reddening and curling, reduced plant vigour, as well as decreased fruit set and size. In apple trees, small fruits with elongated peduncles and enlarged leaf stipules were observed, which suggests AP, although these symptoms are not necessarily indicative of AP. In stone fruit trees, symptoms were less prominent and varied considerably. Leaf rolling and yellowing, or reddening, were mostly observed in apricot and peach, respectively. The development of leaves and flowers late in the vegetative season (autumn bud break) was observed in some samples of sweet and sour cherry, peach and Japanese plum, while proliferation mostly occurred in the European plum and almond. No symptoms, which could indicate phytoplasma presence, were recorded in wild *Prunus* spp. (*P. cerasifera*, *P. mahaleb*, *P. spinosa*, *Prunus* sp.) neighbouring some of the monitored orchards.

3.2 Insect vectors

Five known phytoplasma psyllid vectors, namely *Cacopsylla melanoneura*, *C. picta*, *C. pruni*, *C. pyri* and *C. pyricola* were identified in all surveyed parts of Croatia and throughout the years 2002–2007. Their population density varied and could be correlated to the insecticide treatments performed in the orchard for controlling economically important insect pests (e.g. leaf roller, leaf miner, codling moth, aphids) emerging at the same time in the season as the psyllids. The exception to this observation was *C. pyri*, which is resistant to most insecticides.

AP vector *C. melanoneura* was more widespread and numerous than *C. picta* in apple orchards. Of all psyllid species captured on apples, *C. melanoneura* represented 50.9%, 76.3% and 60.2%, while *C. picta* represented 45.4%, 22% and 38.4% in 2005, 2006, and 2007, respectively. The remainder of psyllid populations in apple consisted of *C. crataegi*, whose share in the populations varied from 1.4-3.7%, depending on the year. However, the geographical distribution of *C. crataegi* was rather limited. It was found only in two locations (Jastrebarsko and Škudelin).

Throughout the monitoring period, *C. pyri* was the most prevalent psyllid in pear (76.4%–80.8%), while the other known PD vector, *C. pyricola*, was present only in 2.4%, 1.7% and 8.5% of the populations in 2005, 2006, and 2007, respectively. A potential PD vector, *C. pyrisuga*, was consistently the second most abundant psyllid in pear, with 19.1%, 21.6% and 10.7% in 2005, 2006 and 2007, respectively. *C. bidens* was found only sporadically in 2005 and 2006, with 1.6% and 0.3%, respectively. As expected, psyllid population density in intensively managed pears was higher, but diversity was lower, than in extensively managed pears. In the intensively managed orchards, the average number of psyllids per orchard collected at one trial was about 200, and consisting mainly of one species (*C. pyri*). In contrast, in extensively managed orchards, the average number of psyllids was 5–10 individuals per orchard, and the diversity was greater.

Relatively low population density of *C. pruni*, a known vector of ESFY, were found in plum, apricot, peach, wild *Prunus cerasifera* and *P. spinosa*. This vector was absent from sweet and sour cherry orchards. The average number of *C. pruni* adults varied per year and per monitoring between 10 and 26 per orchard. The presence of vectors was not monitored in almond orchards.

3.3 Phytoplasma detection and identification

In the first nested PCR assay, 1.2 kb products were used for phytoplasma identification based on the R16F2nR2/*Tru*9I system (Fig. 2). In the case of infection with ribosomal group X phytoplasmas or stolbur alone (Tables 1 and 2), identifying group affiliation was possible solely on the basis of the R16F2nR2/*Tru*9I system. Second nested PCR primed by group specific R16(X)F1/R1, confirmed the identification for ribosomal group X phytoplasmas. PCR amplifications, using primers R16(I)F1/R1 (Fig. 3) specific for ribosomal groups 16SrI, 16SrII and 16SrXII (Lee et al. 1994; Tolu et al. 2006) and digestion of the resulting 1.1 kb long products using *Tru*9I, enabled identification of mixed infections in several samples of fruit trees and vectors yielding additional positives and thus increasing the sensitivity of detection (Tables 1 and 2, Fig. 3).

From 35 out of 78 samples obtained from fruit trees or wild *Prunus* sp. (Table 1), as well as from 10 out of 24 insect samples, phytoplasma 16S rDNA was amplified (Table 2, Fig. 1). Besides single infections, one pear harboured phytoplasmas belonging both to the ribosomal subgroups 16SrX-C and 16SrXII-A, and one Japanese plum harboured phytoplasmas belonging to both the ribosomal subgroups 16SrI-B ('*Ca*. P. asteris', Lee et al. 2002) and 16SrX-B. Phytoplasmas from different subgroups were also found in single or mixed infections in *C. pyri* samples (Table 2, Fig. 3). However, since DNA was extracted from batches of 5 insects, it is also possible that individual insects harboured different phytoplasmas in single infections.

4 Discussion

Management strategies of phytoplasmoses presently rely on disease prevention through quarantine measures, eradication programs, production of pathogen free planting material and chemical control of insect vectors. The implementation of these measures is not possible without sensitive diagnostic tools, and basic knowledge of phytoplasma occurrence in fruit trees and of their potential vectors in the fruit growing areas. Apart from early efforts in identifying phytoplasmoses of apples and pears (Cvjetković 1976; Šarić and Cvjetković 1985), very little was known about the occurrence of phytoplasmoses in fruit trees in Croatia, even though AP, PD and ESFY can be quite devastating diseases. Moreover, there is no data regarding phytoplasma occurrence in fruit tree planting material that is either produced in the country or imported from abroad. The results of a pilot study, investigating the phytoplasma presence by PCR-RFLP in pomatious fruits and their potential psyllids vectors (Križanac et al. 2008), confirmed the occurrence of various '*Candidatus* Phytoplasma' taxa in both plants and psyllids. At the time, only nine pear and three apple orchards were investigated, and insects were collected only in five pear orchards. In view of this and the reports of fruit tree phytoplasma presence in neighbouring countries (Brzin et al. 2003; Ember et al. 2004; Delić et al. 2005; Duduk et al. 2005a; Duduk et al. 2005b), it was deemed important to continue the survey on phytoplasma presence in both pome and stone fruit trees and their potential psyllid vectors.

The occurrence of PD-like symptoms in pear was positively correlated with the occurrence of phytoplasma infections. No

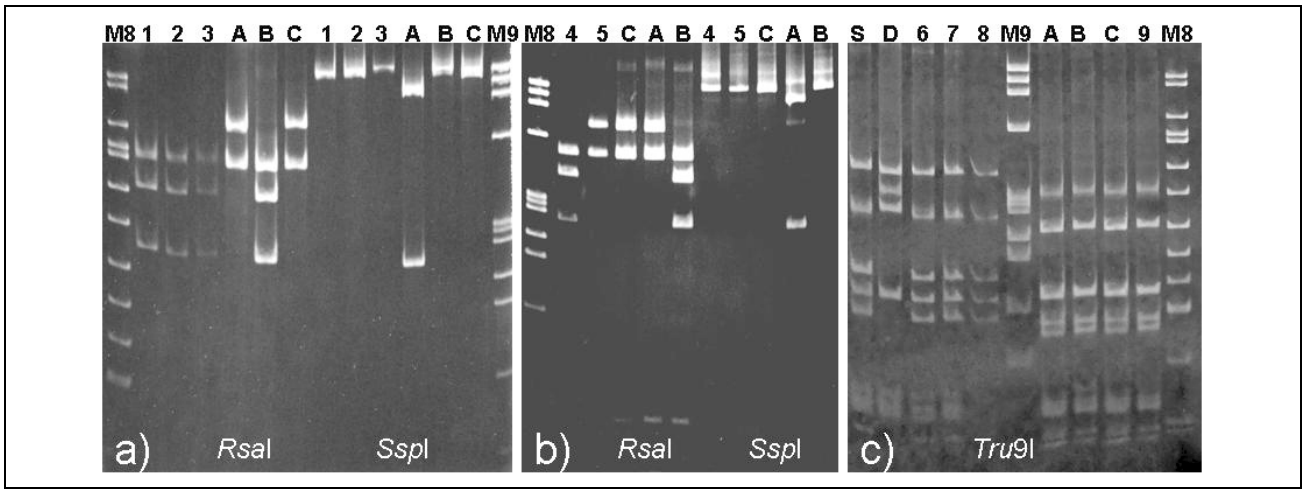

Fig. 2: Representative RFLP patterns of phytoplasma 16S rDNA amplified from various samples by using R16F2nR2 primers. Restriction enzymes used are marked on the corresponding parts of the figure. Reference phytoplasma strains AP-15 ('*Candidatus* Phytoplasma mali', 16SrX-A), GSFY-1 ('*Ca*. P. prunorum', 16SrX-B) and PD ('*Ca*. P. pyri', 16SrX-C) are marked A, B and C, respectively, HYDB ('*Ca*. P. asteris', 16SrI-B) is marked D and SA-1 (16SrXII-A) is S. Panel a) – stone fruit samples from Škudelin: *Prunus salicina* (1), *P. armeniaca* (2), and *Prunus* sp. (3) infected by '*Ca*. P. prunorum'. Panel b) – psyllid samples from Osijek: *Cacopsylla pruni* (4) from European plum infected by '*Ca*. P. prunorum' and *C. pyri* (5) from pear harbouring '*Ca*. P. pyri'. Panel c) – *Pyrus communis* samples from Voloder (6), Škudelin (7) and Jastrebarsko (8) infected by stolbur (16SrXII-A), and one from Jastrebarsko infected by '*Ca*. P. pyri' (9). DNA molecular standards: M8 – pUC Mix Marker (with fragment sizes in base pairs from top to bottom: 1118, 881, 692, 501, 489, 404, 331, 242, 190, 147, 111/110) and M9 – PhiX174, *Hind*III digested (fragment sizes: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118).

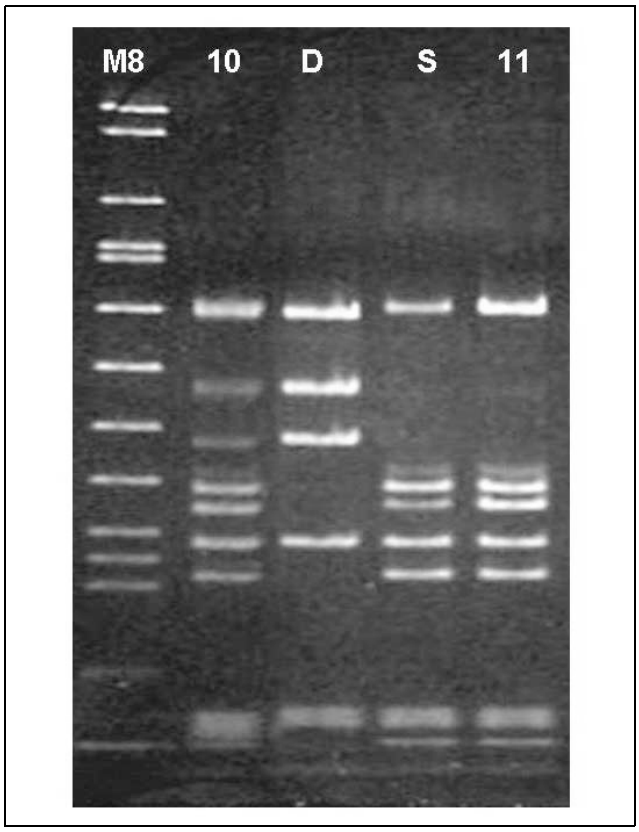

Fig. 3: Representative RFLP patterns of phytoplasma 16S rDNA amplified by using R16(I)F1/R1 primers. Restriction enzyme used is *Tru*9I. Reference phytoplasma strains are D (HYDB, '*Ca*. P. asteris', 16SrI-B) and S (SA-1, 16SrXII-A). For *Cacopsylla pyri* sample (1 batch of 5 individual insects) from Jastrebarsko (10), mixed profile for 16SrI-B and 16SrXII-A phytoplasmas is discernable. In *C. pyri* from Sv. Marija sample (11) only 16SrXII-A phytoplasma pattern is detectable. M8 – the same DNA molecular standard as in Fig. 2.

correlation was found between severity and type of symptoms, and phytoplasma type. Orchards in Jastrebarsko and Voloder that were included in the study by Križanac et al. (2008) were revisited.

A roughly equal proportion of pear samples were infected by either '*Ca*. P. pyri' or stolbur (16SrXII-A). According to our knowledge, this is an unprecedented finding for pear. The relatively high incidence of stolbur in pears, in one sample probably mixed with '*Ca*. P. pyri' (Table 1), indicates that stolbur is common in pears, in contrast to '*Ca*. P. asteris' in a pear in Jastrebarsko (CW region). The finding of stolbur in pear is epidemiologically important because stolbur is widespread in Croatia, especially in vineyards (Šeruga Musić et al. 2009), which are often situated in the vicinity of orchards. Stolbur was not only found in pears, but also in both known PD vectors, *C. pyri* and *C. pyricola*, as well as in the suspected vector *C. pyrisuga* (Table 2). *C. pyri* was investigated in three additional locations (Čiče, St. Petrovo selo, Vučjak Feričanački), and *C. pyrisuga* in two (Osijek and Vučjak Feričanački) with regard to the previous study (Križanac et al. 2008), while other locations were continued to be monitored until 2007. In the psyllid populations obtained from pear, the abundance of *C. pyri* was the highest, averaging 78%, followed by *C. pyrisuga* (17.3%) and *C. pyricola* (4.2%). This trend remained constant throughout the investigation period. Unlike the oligophagous psyllids present in pears (Jarausch and Jarausch 2010), cixiid vectors that transmit stolbur in other crops and weeds are polyphagous (Maixner 2010). One could easily envisage a scenario in which cixiids could transmit stolbur from grapevines and/or weed reservoirs to pear. However, to investigate this was beyond the scope of this study and the epidemiological significance of these findings remains to be investigated.

Phytoplasma infections of apples, so far, could not be ascribed to the common agent '*Ca*. P. mali'. Previously, '*Ca*. P. pyri' and stolbur phytoplasma have been found in a few apple orchards (Križanac et al. 2008). The samples of *C. melanoneura* and *C. picta* from two locations in the CW and CE regions (Table 2) probably did not represent the natural psyllid populations in apple, because sampling was allowed only in a few commercial orchards that were heavily treated with insecticides. Historical AP findings (Šarić and Cvjetković

Table 1: Number and location of plant samples with phytoplasma ribosomal subgroup identification.

Species	Sampling locations (region)	No. positives/tested	Phytoplasma 16Sr subgroup*	No. positives/total
Pear	Čiče (CW)	1/1	X-C + XII-A	
Pyrus Communis L.	Imotski (AD)	1/1	X-C	
	Jastrebarsko (CW)	3/3	X-C; XII-A; I-B	
	St. Petrovo selo (CE)	1/1	XII-A	
	Sv. Marija (CW)	1/1	X-C	
	Škudelin (AD)	2/2	XII-A	
	Varaždin Breg (CW)	1/1	X-C	
	Voloder (CW)	3/3	X-C; 2 XII-A	
	Vučjak Feričanački (CE)	1/1	X-C	14/14
Apple	Kohanjac (CW)	1/1	XII-A	
Malus domestica Borkh.	Šušnjari (CW)	1/1	X-C	
	Varaždin Breg (CW)	1/1	XII-A	3/3
Apricot	Aljmaš (CE)	2/2	X-B	
Prunus armeniaca L.	Baštica (AD)	1/1	XII-A	
	Cres (AD)	1/1	X-B	
	Sladojevci (CE)	0/1	–	
	Škudelin (AD)	2/2	X-B	6/7
Peach	Aljmaš (CE)	0/1	–	
Prunus persica (L.) Batsch	Baštica (AD)	0/1	–	
	Čiče (CW)	1/2	X-B	
	Donji Kraljevec (CW)	0/2	–	
	Erdut (CE)	0/1	–	
	Gornja Trebinja (CW)	1/1	I-B	
	Molvice (CW)	0/1	–	
	Sršići (CW)	1/1	XII-A	
	Staševica (AD)	0/1	–	
	Škudelin (AD)	1/1	XII-A	
	Vučjak Feričanački (CE)	2/2	XII-A; I-B	
	Zagreb (CW)	0/1	–	6/15
European plum	Baštica (AD)	0/1	–	
Prunus domestica L.	Cres (AD)	0/1	–	
	Imotski (AD)	0/1	–	
	Lepoglava (CW)	0/1	–	
	Opatovac (CE)	0/1	–	
	Opuzen (AD)	0/1	–	
	Orahovica (CE)	0/1	–	
	Staševica (AD)	0/1	–	
	Škudelin (AD)	0/1	–	
	Varaždin Breg (CW)	0/1	–	0/10
Japanese plum	Imotski (AD)	0/1	–	
Prunus salicina Lindl.	Kutjevo (CE)	1/1	X-B	
	Sladojevci (CE)	1/1	X-B	
	Škudelin (AD)	1/1	X-B	
	Vučjak Feričanački (CE)	1/1	X-B + I-B	4/5
Almond	Vrana (AD)	0/2	–	
Prunus amygdalus L.	Zemunik (AD)	0/1	–	0/3

Table 1: (Continued)

Species	Sampling locations (region)	No. positives/tested	Phytoplasma 16Sr subgroup*	No. positives/total
Sweet cherry *Prunus avium* L.	Baštica (AD)	0/1	–	
	Cres (AD)	0/3	–	
	Gornja Trebinja (CW)	0/1	–	
	Kaštela (AD)	0/1	–	
	Orahovica (CE)	1/1	XII-A	
	Škudelin (AD)	0/1	–	
	Varaždin Breg (CW)	0/1	–	
	Žeževica (AD)	0/1	–	1/10
Sour cherry *Prunus cerasus* L.	Orahovica (CE)	0/1	–	
	Prkos (AD)	0/2	–	
	Sv. Marija (CW)	0/1	–	0/4
Wild *Prunus* sp.	Cres (AD)	0/3	–	
	Škudelin (AD)	1/1	X-B	
	Vučjak Feričanački (CE)	0/2	–	
	Čiče (CW)	0/1	–	1/7
Total samples:				35/78

* Phytoplasma ribosomal subgroup names separated by ';' indicate individual samples infected by phytoplasmas belonging to different subgroups and those separated by + indicate a mixed infection.

Table 2: Number and location of psyllid samples with phytoplasma ribosomal subgroup identification. Each number represents a batch of 5 individual insects.

Psyllid species	Sampling locations (region)	Plant host	No. positives/tested	Phytoplasma 16Sr subgroup	No. positives/total
Cacopsylla pyri	Čiče (CW)	Pear	0/1	–	
	Jastrebarsko (CW)		1/1	XII-A + I-B	
	Osijek (CE)		2/2	X-C; XII-A	
	St. Petrovo selo (CE)		0/1	–	
	Sv. Marija (CW)		1/1	XII-A	
	Škudelin (AD)		1/2	I-B	
	Voloder (CW)		1/1	X-C + I-B	
	Vučjak Feričanački (CE)		0/1	–	6/10
C. pyricola	Jastrebarsko (CW)	Pear	1/2	XII-A	1/2
C. pyrisuga	Jastrebarsko (CW)	Pear	1/2	X-C	
	Osijek (CE)		0/1	–	
	Voloder (CW)		1/1	XII-A	
	Vučjak Feričanački (CE)		0/1	–	2/5
C. picta	Jastrebarsko (CW)	Apple	0/1	–	
	Trnjani (CE)		0/1	–	0/2
C. melanoneura	Obreška (CW)	Apple	0/1	–	
	Trnjani (CE)		0/1	–	0/2
C. pruni	Jastrebarsko (CW)	European plum	0/1	–	
	Osijek (CE)		1/1	X-B	
	Škudelin (AD)	Apricot	0/1	–	1/3
Total samples:					10/24

* Phytoplasma ribosomal subgroup names separated by ';' indicate individual samples infected by phytoplasmas belonging to different subgroups and those separated by + indicate a mixed infection.

1985) together with recent AP reports in neighbouring states (Brzin et al. 2003; Delić et al. 2005; Duduk et al. 2008) and the occurrence of known AP-vectors, *C. melanoneura* and *C. picta*, in surveyed apple orchards, can be taken as strong indications of AP presence in Croatia. The observed higher abundance of *C. melanoneura* compared to *C. picta* in apple poses an interesting starting point for epidemiological and/or insecticide resistance investigations. The recent increase in apple production in the country, the predominant use of local planting material and the scarcity of molecular data on phytoplasma identity, call for the continuation of the study in apples and AP vectors before drawing relevant epidemiological and disease management conclusions.

In the framework of this study, 61 new samples of stone fruits and three samples of *C. pruni* were analysed. Phytoplasmas could not be identified in European plum, almond or sour cherry even though samples were taken from plants with symptoms potentially indicative of phytoplasmoses. Due to the rigorous detection system that included a second round of nested PCR with group specific R16(X)F1/R1 in addition to the more general R16(I)F1/R1 primers, we can exclude the possibility that phytoplasmas at very low concentrations were missed. This suggests that symptoms in plum, almonds, and sour cherry could have been caused by other biotic or abiotic agents. Only one sweet cherry sample from CE region harboured stolbur phytoplasma (Table 1). Tree decline (Paltrinieri et al. 2008) or other severe symptoms that could specifically be associated with phytoplasma presence were not observed in sweet cherry. The ESFY agent '*Ca*. P. prunorum' was identified in its main vector *C. pruni*, in one asymptomatic wild *Prunus*, and in the majority of symptomatic apricot and Japanese plum samples, but only in one peach (Fig. 1; Table 1). In five more peach samples stolbur (16SrXII-A) and aster yellows (16SrI-B) phytoplasmas were identified which was unusual, but not unprecedented for these hosts (Paltrinieri et al. 2001; Duduk et al. 2008). With three phytoplasma taxa, peach hosts the most types of phytoplasmas of all the stone fruittrees, whilst apricot and Japanese plum follow by hosting two, sometimes in mixed infection (Table 1). This is in accordance with the postulated higher susceptibility of the three above-mentioned hosts to phytoplasma infections (Carraro et al. 1998b). As yet, phytoplasma infection in peach, apricot or Japanese plum has not resulted in a severe disease development that could potentially endanger local production.

The results presented here suggest the widespread occurrence of '*Ca*. P. pyri' and '*Ca*. P. prunorum' in Croatia, the most important pear and stone fruit phytoplasma agents, respectively, as well as their psyllids vectors. This calls for implementation of comprehensive control strategies for fruit tree phytoplasmas and their vectors. High incidence and wide geographical distribution of stolbur phytoplasma in pears suggests its involvement in pear decline in Croatia. This is also true for a suspected new '*Ca*. P. pyri' vector, *C. pyrisuga*, that harboured stolbur phytoplasma. The epidemiological relevance of the latter two findings, as well as the epidemiology of phytoplasmoses in apples needs to be investigated further.

Acknowledgements

The Croatian Ministry of Agriculture, Forestry and Water Management financed this research. The technical assistance of Ana Babić and Ana Čalija is gratefully acknowledged. This work is dedicated to our deceased colleague and friend Ivan Mikec (1948–2010).

References

Angelini, E., D. Clair, M. Borgo, A. Bertaccini, E. Boudon-Padieu, 2001: Flavescence dorée in France and Italy – occurrence of closely related phytoplasma isolates and their near relationships to Palatinate grapevine yellows and an alder yellows phytoplasma. Vitis **40**, 79-86.

Bertaccini, A., 2003: URL: http://137.204.42.130/person/collection-september_2003.pdf. Date of last accession: June 8, 2004.

Bertaccini, A., 2007: Phytoplasmas: diversity, taxonomy, and epidemiology. Front. Biosci. **12**, 673-689.

Brzin, J., P. Ermacora, R. Osler, N. Loi, M. Ravnikar, N. Petrovič, 2003: Detection of apple proliferation phytoplasma by ELISA and PCR in growing and dormant apple trees. J. Plant Dis. Protect. **110**, 476-483.

Carraro, L., N. Loi, P. Ermacora, A. Gregoris, R. Osler, 1998a: Transmission of pear decline by using naturally infected *Cacopsylla pyri*. Acta Hort. **472**, 665-668.

Carraro, L., R. Osler, N. Loi, P. Ermacora, E. Refatti, 1998b: Transmission of European stone fruit yellows phytoplasma by *Cacopsylla pruni*. J. Plant Pathol. **80**, 233-239.

Cvjetković, B., 1976: Decay of pears in Dalmatia. Biljna zaštita **3**, 104-105.

Cvjetković, B., I. Mikec, V. Kajić, 1987: Apple proliferation. Glasnik zaštite bilja **9**, 349-351.

Delić, D., M. Martini, P. Ermacora, L. Carraro, A. Myrta, 2005: First report of fruit tree phytoplasmas and their psyllid vectors in Bosnia and Herzegovina. J. Plant Pathol. **87**, 150.

Duduk, B., S. Botti, V. Trkulja, M. Ivanović, J. Stojčić, A. Bertaccini, 2005a: Occurrence of pear decline in Bosnia and Herzegovina. J. Plant Pathol. **87**, 75.

Duduk, B., M. Ivanović, A. Obradović, S. Paltinieri, A. Bertaccini, 2005b: First report of pear decline phytoplasmas on pear in Serbia. Plant Dis. **89**, 774.

Duduk, B., M. Ivanović, S. Paltrinieri, A. Bertaccini, 2008: Phytoplasmas infecting fruit trees in Serbia. Acta Hort. **781**, 351-358.

Ember, I., M. Nemeth, L. Krizbai, M. Kolber, M. Szakal, S. Botti, A. Bertaccini, G.Y. Bohar, R. Zsovak-Hangyal, 2004: Identification of phytoplasmas on pomaceous fruit tree species in Hungary. Acta Hort. **657**, 443-448.

Frisinghelli, C., L. Delaiti, M.S. Grando, D. Forti, M.E. Vindimian, 2000: *Cacopsylla costalis* (Flor 1861), as a vector of apple proliferation in Trentino. J. Phytopathol. **148**, 425-431.

Gundersen, D.E., I.-M. Lee, S.A. Rehner, R.E. Davis, D.T. Kingsbury, 1994: Phylogeny of mycoplasmalike organisms (phytoplasmas): a basis for their classification. J. Bacteriol. **176**, 5244-5254.

Gundersen, D.E., I.-M. Lee, 1996: Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. Phytopathol. Mediterr. **35**, 144-151.

Hogenhout, S.A., K. Oshima, E.D. Ammar, S. Kakizawa, H.N. Kingdom, S. Namba, 2008: Phytoplasmas: bacteria that manipulate plants and insects. Mol. Plant. Pathol. **9**, 403-423.

IRPCM Phytoplasma/Spiroplasma Working Team–Phytoplasma taxonomy group, 2004: '*Candidatus* Phytoplasma', a taxon for the wall-less, non-helical prokaryotes that colonize plant phloem and insects. Int. J. Syst. Evol. Microbiol. **54**, 1243-1255.

Jarausch, B., N. Schwind, W. Jarausch, G. Krczal, E. Dickler, E. Seemüller, 2003: First report of *Cacopsylla picta* as a vector of apple proliferation phytoplasma in Germany. Plant Dis. **87**, 101.

Jarausch, B., W. Jarausch, 2010: Psyllid vectors and their control. In: P.G. Weintraub, P. Jones (eds.): Phytoplasmas: genomes, plant hosts and vectors, pp. 250-271. CAB International, Wallingford, United Kingdom.

Jensen, D.D., W.H. Griggs, C.Q. Gonzales, H. Schneider, 1964: Pear decline virus transmission by pear psylla. Phytopathology **54**, 1346-1351.

Križanac, I., I. Mikec, Ž. Budinščak, M. Šeruga Musić, M. Krajačić, D. Škorić, 2008: Pomaceous fruit tree phytoplasmas and their potential vectors in Croatia. Acta Hort. **781**, 477-482.

Kube, M., B. Schneider, H. Kuhl, T. Dandekar, K. Heitmann, A.M. Migdoll, R. Reinhardt, E. Seemüller, 2008: The linear

chromosome of the plant pathogenic mycoplasma '*Candidatus* Phytoplasma mali'. BMC Genomics **9**: 306 (doi: 10.1186/1471-2164-9-306).

Kučerova, J., L. Talacko, P. Lauterer, M. Navratil, R. Fialova, 2007: Molecular tests to determine '*Candidatus* Phytoplasma pyri' presence in psyllid vectors from a pear tree orchard in the Czech Republic – a preliminary report. Bull. Insectol. **60**, 191-192.

Lee, I.-M., D.E. Gundersen, R.W. Hammond, R.E. Davis, 1994: Use of mycoplasmalike organism (MLO) group-specific oligonucleotid primers for nested-PCR assays to detect mixed-MLO infections in a single host plant. Phytopathology **84**, 559-566.

Lee, I.-M., A. Bertaccini, M. Vibio, D.E. Gundersen, 1995: Detection of multiple phytoplasmas in perennial fruit trees with decline symptoms in Italy. Phytopathology **85**, 728-735.

Lee, I.-M., R.E. Davis, D.E. Gundersen-Rindal, 2000: Phytoplasma: phytopathogenic Mollicutes. Annu. Rev. Microbiol. **54**, 221-255.

Lee, I.-M., D.E. Gundersen-Rindal, R.E. Davis, K.D. Bottner, C. Marcone, E. Seemüller, 2002: '*Candidatus* Phytoplasma asteris', a novel phytoplasma taxon associated with aster yellows and related diseses. Int. J. Syst. Evol. Microbiol. **54**, 1037-1048.

Maixner, M., 2010: Phytoplasma epidemiological systems with multiple plant hosts. In: P.G. Weintraub, P. Jones (eds.): Phytoplasmas: genomes, plant hosts and vectors, pp. 213-232. CAB International, Wallingford, United Kingdom.

Paltrinieri, S., M. Martini, E. Stefani, M. Pondrelli, C. Fideghelli, A. Bertaccini, 2001: Phytoplasma infection in peach and cherry in Italy. Acta Hort. **550**, 365-370.

Paltrinieri, S., A. Bertaccini, C. Lugaresi, 2008: Phytoplasmas in declining cherry plants. Acta Hort. **781**, 409-415.

Seemüller, E., B. Schneider, 2004: '*Candidatus* Phytoplasma mali', '*Candidatus* Phytoplasma pyri' and '*Candidatus* Phytoplasma prunorum', the causal agents of apple proliferation, pear decline and European stone fruit yellows, respectively. Int. J. Syst. Evol. Microbiol. **54**, 1217-1223.

Steiner, H., 1967: Die Anwendungsmöglichkeiten der Klopfmethode bei Arbeiten über die Obstbaumfauna. Entomophaga H. S. **3**, 17-20.

Šarić, A., B. Cvjetković, 1985: Mycoplasma-like organism associated with apple proliferation and pear decline- like disease of pears. Agric. Conspec. Sci. **68**, 61-67.

Šeruga, M., D. Škorić, S. Botti, S. Paltrinieri, N. Juretić, A.F. Bertaccini, 2003: Molecular characterization of a phytoplasma from the aster yellows (16SrI) group naturally infecting *Populus nigra* L. 'Italica' trees in Croatia. Forest Pathol. **33**, 113-125.

Šeruga Musić, M., D. Škorić, Ž. Budinščak, I. Križanac, I. Mikec, 2009: Survey of phytoplasma diversity in heavily grapevine yellows affected areas of Croatia. Extended abstractsm 16th Meeting ICVG, Dijon, France, 31 Aug – 4 Sep, 2009, Le Progrès Agricole et Viticole, **Hors Série**, 206-207.

Tedeschi, R., C. Visentin, A. Alma, D. Bosco, 2003: Epidemiology of apple proliferation (AP) in northwestern Italy: evaluation of the frequency of AP-positive psyllids in naturally infected populations of *Cacopsylla melanoneura* (Homoptera: Psyllidae). Ann. Appl. Biol. **142**, 285-290.

Tolu, G., S. Botti, R. Garau, V.A. Prota, A. Sechi, U. Prota, A. Bertaccini, 2006: Identification of a 16SrII-E Phytoplasma in *Calendula arvensis*, *Solanum nigrum* and *Chenopodium* spp. Plant Dis. **90**, 325-330.