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DETECTION OF EUROPEAN FRUIT TREE PHYTOPLASMAS AND THEIR INSECT VECTORS IN IMPORTANT FRUIT-GROWING REGIONS IN BULGARIA

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Abstract

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Apple proliferation (AP), caused by '*Candidatus* Phytoplasma mali', European stone fruit yellows (ESFY), caused by '*Candidatus* Phytoplasma prunorum' and Pear decline (PD) caused by '*Candidatus* Phytoplasma pyri' are one of the most important fruit tree diseases in Europe, but their distribution in Bulgaria is not well known. During 2010 – 2013, the presence and the spread of the fruit tree phytoplasmas and their vectors were investigated in one of the major fruit tree growing regions in Bulgaria. All psyllid species described as vectors of fruit tree phytoplasmas were present in the investigated areas. Phytoplasma infection in plant and psyllid samples was analyzed by universal and specific PCR. A total of 86 plant samples and 1569 insect samples obtained from 11 different sites were tested. As result, all three phytoplasmas were detected in their hosts and vectors in five different geographical regions of Bulgaria. This is the first report of '*Ca.* P. mali' and '*Ca.* P. pyri' in Sofia region as well as of '*Ca.* P. prunorum' in Sofia and Kjustendil regions. This is also the first report of a '*Ca.* P. pyri' detection in *Cacopsylla bidens* which might be a further vector of PD.

Key words: '*Candidatus* Phytoplasma mali', '*Candidatus* Phytoplasma prunorum', '*Candidatus* Phytoplasma pyri', apple proliferation, European stone fruit yellows, pear decline, PCR detection, psyllid vectors

Introduction

In recent years, phytoplasma diseases have become one of the most important diseases of fruit trees in Bulgaria but until now the existence and the spread of fruit tree phytoplasmas and their vectors have not been studied in detail. Apple proliferation (AP) is caused by '*Candidatus* Phytoplasma mali', '*Candidatus* Phytoplasma prunorum' is the agent of Apricot chlorotic leafroll mycoplasma (European stone fruit yellows – ESFY) and '*Candidatus* Phytoplasma pyri' is the agent of Pear decline (PD) (Seemüller and Schneider, 2004; Jarausch and Jarausch, 2010). Phytoplasmas are cell wall-less Gram-positive bacteria of the class *Mollicutes*, and both their cell and genome size are the smallest among bacteria. Phytoplasmas are restricted to the phloem of infected plants and are naturally transmitted by phloem-feeding vectors – in the case of European fruit tree phytoplasmas psyllids of the family Psyllidae. However, phytoplasmas can also be transmitted through infected propagating material (Bertaccini, 2007). Because of the high risk of phytoplasma spreading on new territories, the three fruit tree phytoplasmas are still on the EU and EPPO quarantine lists, and are under special regulations (Council Directive 2000/29/EC; EPPO A2 list). In Bulgaria, '*Ca.* P. prunorum' and '*Ca.* P. pyri' have been first detected near the town of Plovdiv (Topchiiska et al., 2000; Topchiiska and Sakalieva, 2001, 2002). In 1960, AP has been reported to occur in Bulgaria by Blattny, Sen and Blattny Jun, ref. by Trifonov (1972). In 2012, the first summarized results of the official phytosanitary monitoring program during the period 2007 – 2011 of the Bulgarian Food Safety Agency (BFSA) were published (Etropolska and Laginova, 2012).

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A few species of the psyllid genus *Cacopsylla* (Hemiptera: Psyllidae) have been demonstrated to be vectors of European fruit tree phytoplasmas. Two psyllids, *Cacopsylla picta* (Foerster) and *Cacopsylla melanoneura* (Foerster) are recognized vectors of '*Ca.* P. mali'. The psyllid *Cacopsylla pruni* Scopoli was described as vector of '*Ca.* P. prunorum' whereas three psyllid species also of the genus *Cacopsylla* are recognized or presumed vectors of '*Ca.* P. pyri': *Cacopsylla pyri* (Linnaeus), *C. pyricola* (Foerster) and *C. pyrisuga* (Foerster), reviewed by Jarausch and Jarausch (2010).

All those *Cacopsylla* species have been described for Bulgaria by psyllid taxonomists in the 60s of the last century (Harizanov, 1966a, 1966b, 1982), long before they have been identified as phytoplasma vectors. As phytoplasma vectors, they have never been studied in Bulgaria. In 2011, we published the first report about PCR detection of '*Ca.* P. prunorum' in individuals of their vector *Cacopsylla pruni* from two geographically different regions – Sofia and Kjustendil districts (Etropolska et al., 2011a).

However, up to the beginning of our work in 2010 almost nothing was known about the incidence and the spread of these quarantine diseases and their vectors in Bulgaria. Therefore, the objective of this study was to elucidate the actual status of European fruit tree phytoplasma infections in Bulgaria as well as the presence of the known or putative insect vectors.

Materials and Methods

Sampling of plant and insect material

A survey was conducted from the end of August till the end of October during the period 2010 - 2013 in 11 different sampling sites with conventional or uncultivated fruit tree orchards as well as in nurseries in six different districts in Bulgaria. The sampling sites are given in Table 1 and Table 2.

Plant material was derived from symptomatic and non-symptomatic trees from *Malus, Prunus* and *Pyrus spp.* in autumn when the concentration of the phytoplasma in the upper parts of the tree is highest.

Insects were caught using sweep-netting during the period March-July of each year. Captured psyllids were frozen at -20°C and psyllid species identification was done using different determination keys (Hodkinson and White, 1979; Ossiannilsson, 1992; Burckhardt and Jarausch, 2007). In 2013, some of the species were confirmed by Dr. Daniel Burckhardt from Naturhistorisches Museum, Basel, Switzerland.

Molecular test for phytoplasma infection in plants and insects

Phloem was prepared from branches of plant samples and was extracted with a modified CTAB-based protocol as described by Jarausch et al. (2011). After the morphological identification, DNA was extracted from single psyllid indi-

Table 1
Total number of plants samples, analyzed for European fruit tree phytoplasmas with universal and specific primers

Host plant	Location	Number of Samples	Phytoplasma type	PCR positive/ total number of samples			
				fU5/rU4	ECA1/ ECA2	fPD/ rPD	AP3/ AP4
M. domestica	PL, DA1, PH, SOF1	29	'*Ca.* P. mali'	24/29	-	-	24/24
P. domestica	DA2, SOF1,	27	'*Ca.* P. prunorum'	18/27	16/18	-	-
P. armeniaca	PH	2	'*Ca.* P. prunorum'	0/2	-	-	-
P. persica	SL	10	'*Ca.* P. prunorum'	2/10	0/2		
P. persica	PH	1	'*Ca.* P. prunorum'	0/1	-	-	-
P. comunnis	DA2, PH, SOF1	17	'*Ca.* P. pyri'	15/17	-	14/17	-
Total:		86					

PL - Plovdiv (Zelenikovo village, Plovdivski region); DA1 - Dupnica 1 (Piperovo village, Kjustendilski region); DA2 Dupnica 2 (Djakovo village, Kjustendilski region); SOF1 - Sofia 1 (Gorni Lozen village, Sofiiski region); SL - Sliven (Dotluka, Slivenski region); PH - Petrich (Rupi village, Blagodevgradski region).

Table 2
Total number of insects samples, analyzed for European fruit tree phytoplasmas with universal and specific primers

Insect species	Host plant	Location	N° of samples	Phyto-plasma type	PCR positive/ total number of samples			
					fU5/ P7	ECA1/ ECA2	fPD/ rPD	AP3/ AP4
C. pruni	*P. domestica*	DA2, SOF1, SOF2, KL1	565	'*Ca.* P. prunorum'	13/565	13/13	-	-
C. picta	*M. domestica*	DA1, DA2, KL1, KL2, SOF1, SOF2, SOF3	71	'*Ca.* P. mali'	2/71	-	-	2/2
C. melanoneura	*M. domestica*	DA2, SOF1, PL	233	'*Ca.* P. mali'	0/233	-	1/233	0/1
C. affinis	*Crataegus spp*	DA2, SOF1	42	'*Ca.* P. mali'	0/42	-	-	-
C. mali	*M. domestica*	SOF1, KL1	1	'*Ca.* P. mali'	0/1	-	-	-
C. pyri	*P. comunnis*	SOF1, SOF4, PH, KL1, KL2	358	'*Ca.* P. pyri'	1/358	-	1/1	-
C. pyricola	*P. comunnis*	DA2, SOF1, SOF3, KL1	82	'*Ca.* P. pyri'	2/82	-	2/2	-
C. pyrisuga	*P. comunnis*	DA2, KL1, KL2, SOF1, SOF4	189	'*Ca.* P. pyri'	0/189	-	-	-
C. bidens	*P. comunnis*	DA2, KL1, SOF1	28	'*Ca.* P. pyri'	1/28	-	1/1	-
Total:			**1569**					

PL - Plovdiv (Zelenikovo village, Plovdivski region); DA2 Dupnica 2 (Djakovo village, Kjustendilski region); KL1 – (Jabulkovo village, Kjustendilski region); KL2 (Nikolichevci village, Kjustendilski region); PH - Petrich (Rupi village, Blagodevgradski region); SOF1 - Sofia 1 (Gorni Lozen village, Sofiiski region); SOF2 – Sofia (Pancharevo, Sofiiski region); SOF3 – Sofia (Simeonovo village, Sofiiski region); SOF4 – Sofia (Vragdebna village, Sofiiski region)

viduals with a modified CTAB-based protocol as described by Jarausch et al. (2011) or by the TNES protocol of Sauvion (2012).

PCR amplification of phytoplasma DNA was achieved with universal ribosomal primers: fU5/rU4 in the plant samples (Lorenz et al., 1995) and fU5/rP7 in the insect samples (Lorenz et al., 1995; Schneider et al., 1995). All positive samples were tested with the group specific primers fO1/rO1 (Lorenz et al., 1995). For the confirmation of '*Ca.* P. mali' the specific primers AP3/AP4 (Jarausch et al., 1994) were used; for the confirmation of '*Ca.* P. prunorum', the ESFY-specific non-ribosomal primers ECA1/ECA2 were applied (Jarausch et al., 1998) and '*Ca.* P. pyri' was identified with the specific primer pair fPD/rPD developed by Etropolska et al. (2011b).

Results and Discussion

During the survey period from 2010 to 2013, AP, ESFY and PD were found in commercial or uncultivated orchards in five of the most important fruit-growing regions of Bulgaria: Sofiiski, Blagoevgradski, Plovdivski, Kjustendilski and Slivenski region. Infected apple trees showed typical symptoms of AP, like 'witches' - broom' and enlarged stipules in all four regions. Typical symptoms of ESFY like preliminary blooming in late winter, apricot and peach chlorotic leaf roll, plum leptonecrosis and decline of branches or entire trees were found in Sofiiski, Kjustendilski and Slivenski region. Pear trees with small, leathery and up-rolled leaves, abnormal leaf reddening and premature leaf drop, or decline of branches and trees were the most common symptoms of PD in Sofiiski, Kjustendilski and Blagoevgradski region. In total, 86 plant samples were tested by PCR for the presence of European fruit tree phytoplasmas. As shown in Table. 1, phytoplasmas were detected in most of the samples using universal phytoplasma detection primers. The only exception was peach which had low infection rates. However, this has already been reported from Germany where in particular in peach no good correlation between symptoms and PCR detection of the phytoplasma was obtained (Jarausch et al., 2008). Specific identification primers were used to confirm

the presence of '*Ca.* P. mali' in 24 apple samples, the presence of '*Ca.* P. prunorum' in 16 European plum samples and the identity of '*Ca.* P. pyri' in 14 pear samples.

In the surveyed fruit tree orchards all known and putative psyllid vectors of fruit tree phytoplasmas were identified: *C. pruni, C. picta, C. melanoneura, C. affinis, C. pyri, C. pyrisuga*, *C. pyricola* and *C. bidens.* As shown in Table 2, more than 1500 individuals of the *Cacopsylla* species were collected in the different fruit tree orchards and individually analyzed by PCR for the presence of phytoplasmas. In a total of 565 individuals tested, '*Ca.* P. prunorum' was identified in 13 specimen of its known vector *Cacopsylla pruni*. This represents an infection rate of 2.3% which is in the range of the infection rates reported from Germany and France, were annually 2 - 3% of *C. pruni* were found infected (Jarausch et al., 2001; Jarausch et al, 2007, 2007b).

'Ca. P. mali' was found only in its acknowledged vector *Cacopsylla picta* which is considered as main vector of AP (Jarausch et al., 2011). It was not found in *Cacopsylla melanoneura* which is the most important vector only in Northwestern Italy (Tedeschi et al., 2002; Tedeschi and Alma, 2004) but not in Germany (Mayer et al., 2009). *C. melanoneura* was found in mixed population with *C. picta* on *Malus*, and also in mixed population with *C. affinis* on *Crataegus* spp. One out of 233 individuals was tested positive for a phytoplasma which could be identified with specific primers as '*Ca*. P. pyri'. As PD-infected pear trees were in the neighborhood of the *C. melanoneura* sampling site it cannot be ruled out that this individual acquired the phytoplasma there. So far, it is not known whether *C. melanoneura* can be a vector of '*Ca.* P. pyri'. The only way to proof this hypothesis is through the method of transmission trials (Jarausch and Jarausch, 2010).

In a total of 71 individuals of *C. picta*, two of them were found infected with '*Ca*. P. mali'. The percentage of infection is 2.8%, which is very low in comparison with other regions like Germany and South Tirol where every year the percent of infection is around 10% (Baric et al., 2010; Jarausch et al., 2011).

In a total of 358 tested individuals of the main vector *Cacopsylla pyri* (Carraro et al., 1998), only one individual was confirmed to be infected with *'Ca.* P. pyri', and the percentage of infection is 0.28% respectively. Infection with *'Ca.* P. pyri' was found also in two individuals out of 82 tested of the other main vector *Cacopsylla pyricola* (Foerster) (Davies et al., 1992). The percentage of infection is around 2.4%. In comparison, in England this percentage is around 17% (Davies and Eyre, 1996). In addition two other *Cacopsylla* species, *Cacopsylla pyrisuga* and *Cacopsylla bidens*, were found in some of the observed pear orchards (Table 2). More than 180 individuals of *Cacopsylla pyrisuga* were tested, but no phytoplasma infection was detected. *Cacopsylla bidens* is reported by Burckhardt and Jarausch (2007) and Jerinič–Prodanovič (2010, 2011) to be present in Bulgaria and in some neighboring countries, but for Bulgaria any additional information about the distribution and the biology of this species is missing. In our study *Cacopsylla bidens* was found in three different pear orchards (Table 2). Furthermore, one out of 28 individuals was confirmed to be infected with *'Ca.* P. pyri'. The calculated infection rate of 3.6% would be the highest for all putative PD vectors in Bulgaria. To our knowledge, this species has never been tested for its phytoplasma vectoring capacity. Thus, our result is the first report of an infection of *C. bidens* with *'Ca*. P. pyri'. Transmission trials with this species are now needed to conclude if *C. bidens* is a vector of *'Ca.* P. pyri' in Bulgaria.

- ***Distribution of 'Ca. P. mali', the agent of Apple proliferation, in Bulgaria***

AP was found in our survey in the following districts of Bulgaria: Plovdiv, Kjustendil, Sofia and Blagoevgrad (Figure 1). '*Ca*. P. mali' was confirmed in Veliko Turnovo district as well (Etropolska and Laginova, 2012). Infected individuals of *Cacopsylla picta* were found in Kjustendil and Sofia districts. This is the first confirmation of '*Ca.* P. mali' on the territory of Sofia districts.

In total, the results of our survey and from the data of the official monitoring program of BFSA showed that AP is spread on the territory of 5 out of all 28 districts in Bulgaria.

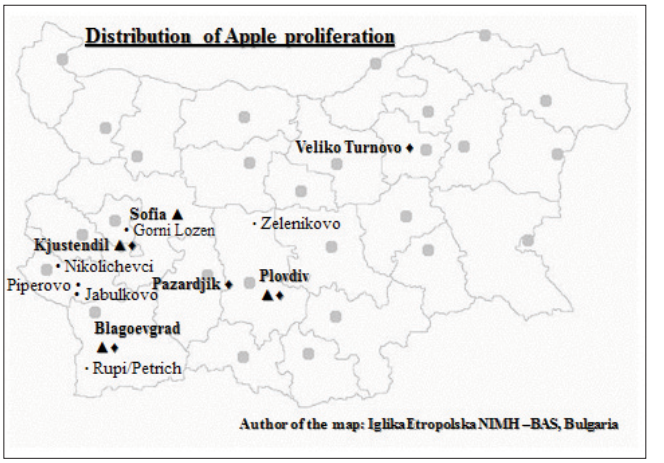

Fig. 1. Distribution of '*Candidatus* Phytoplasma mali' "▲"- Detection of AP per region in our survey; "♦" - Detection of AP per region by the official control (BFSA)

- ***Distribution of 'Ca. P. prunorum', the agent of Apricot chlorotic leafroll (European stone fruit yellows – ESFY), in Bulgaria***

ESFY was found for first time in insect samples collected in orchards on the territory of Sofia (Gorni Lozen) and Kjustendil (Djakovo village) districts (Etropolska et al., 2011a) (Figure. 2). In 2012 we confirmed the presence of ESFY in plant samples on the territory of Sliven district. The published data from the official monitoring showed that ESFY is present also in Burgas, Pazardjik, Dobrich and Yambol districts (Etropolska and Laginova, 2012). Together with our results ESFY is confirmed in 7 out of the 28 districts in Bulgaria.

- ***Distribution of 'Ca. P. pyri', the agent of Pear decline, in Bulgaria***

PD was confirmed in plant samples from Kjustendilski and Blagoevgradski regions. Infection was confirmed also in insects from two geographically isolated orchards on the territory of Sofia district. This is the first report of '*Ca*. P. pyri' in Sofia district.

Together with the data of BFSA, this phytoplasma is present on the territory of 8 out of 28 districts of Bulgaria (Figure 3).

Conclusion

In conclusion all three European fruit tree phytoplasmas '*Ca*. Phytoplasma mali' (apple proliferation), '*Ca*. Phytoplasma pyri' (pear decline) and '*Ca*. Phytoplasma prunorum' (European stone fruit yellows) are present in Bulgaria. This work is the first report of '*Ca*. P. mali' and '*Ca*. P. pyri' in Sofia region. In our survey '*Ca*. P. prunorum' was found for the first time in spring of 2011 in Sofia and Kjustendil regions (Etropolska et al. 2011a). Infection was confirmed in samples from symptomatic and non-symptomatic trees and also in the psyllid species known as main or putative fruit tree phytoplasma vectors.

From all three phytoplasmas, pear decline is the most spread phytoplasma disease in Bulgaria. However, important questions remain open: where is the origin of the phytoplasma infections or how long do they already exist on the Bulgarian territory? Which are the main vectors of the respective phytoplasma diseases in Bulgaria? Molecular approaches of phytoplasma characterization and specific transmission trials with candidate vector species are needed to answer these questions.

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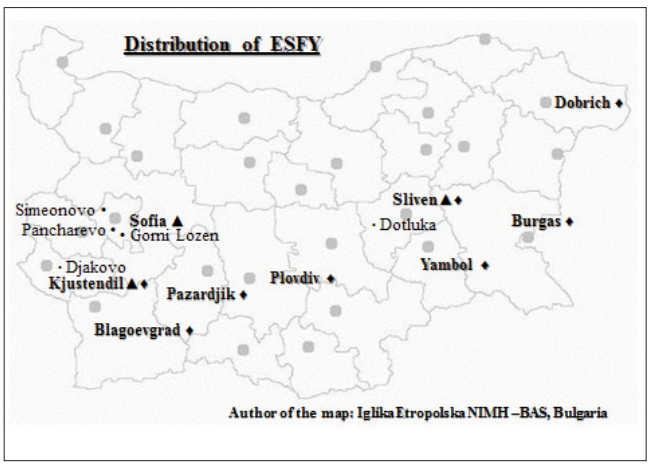

Fig. 2. Distribution of '*Candidatus* Phytoplasma prunorum' "▲"- Detection of ESFY per region in our survey; "♦" - Detection of ESFY per region by the official control (BFSA)

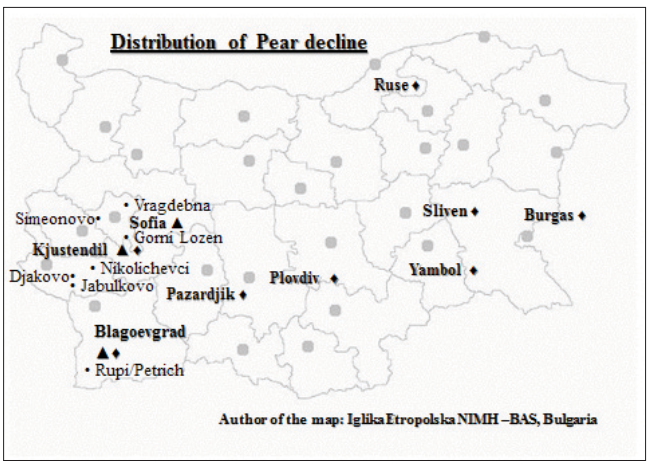

Fig. 3. Distribution of '*Candidatus* Phytoplasma pyri' "▲"- Detection of PD per region in our survey; "♦" - Detection of PD per region by the official control (BFSA)

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