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Survey on the occurrence and infection status of *Cacopsylla pruni*, vector of European stone fruit yellows in Hungary

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Abstract

This study aimed to obtain data on the seasonal activity, preferred host plants, and phytoplasma infection status of the psyllid *Cacopsylla pruni* (Scopoli), vector of European stone fruit yellows (ESFY) phytoplasma ('*Candidatus* Phytoplasma prunorum') in Hungary. Individuals of *C. pruni* were found throughout the whole collection period at all localities studied. In the year 2014 the reimmigrants were found on *Prunus* from March to May and the new generation from May to June, with a peak of the population at the end of April. The preferred host plants appeared to be myrabolan (*P. cerasifera*), followed by blackthorn (*P. spinosa)*, and other *Prunus* species. Ratio of male and female individuals varied in the collection period, with a substantial decrease of male presence in case of reimmigrants. Concerning phytoplasma infection of the species captured in the studied orchards, our results showed that individuals of *C. pruni* were infected by '*Ca.* P. prunorum' in the overwintering as well as in the new generation. The ratio of the ESFY infected psyllids was uniformly 15% in the males and females, and slightly higher (16%) in the nymphs. Molecular classification of *C. pruni* individuals by the ITS primer set 3 assigned unambiguously all the collected *C. pruni* specimens into genetic group B.

Key words: psyllid, vector, *Cacopsylla pruni*, ESFY, '*Candidatus* Phytoplasma prunorum'.

Introduction

European stone fruit yellows (ESFY) (Lorenz *et al.*, 1994) caused by '*Candidatus* Phytoplasma prunorum' (Seemüller and Schneider, 2004) is prevalent in the most important stone fruit production areas of Central and Southern Europe. It causes substantial impact in apricots, Japanese plums and peaches (Marcone *et al.*, 2010), but also affects different *Prunus* species. It is one of the most important diseases of apricot on several growing sites in Hungary (Tarcali and Kövics, 2012).

This pathogen can be transmitted and spread by propagation material and the psyllid vector, *Cacopsylla pruni* (Scopoli), the vector of '*Ca.* P. prunorum' (Carraro *et al.*, 1998). *C. pruni* is an univoltine psyllid, strictly oligophagous on *Prunus*, and widespread in Europe (Marcone *et al*., 2010). In spring, eggs of *C. pruni* are laid by overwintering adults on *Prunus* spp. Individuals of new generations develop on *Prunus* spp. through five nymphal stages into adults, and new adults abandon the reproduction host in summer to overwinter on conifers (Ossiannilsson, 1992; Thébaud *et al.*, 2009). Studies suggest that *C. pruni* individuals returning to *Prunus* spp. in spring (reimmigrants) are the most efficient disease vectors. A secondary spread of ESFY within the orchard during the vegetation period seems to have less significance (Carraro *et al*., 2004; Thébaud *et al*., 2009). Population dynamics, mechanism of transmission and transmission efficiency of *C. pruni* have been studied intensively in several European countries (Carraro *et al.*, 2001; Jarausch *et al.*, 2001; Steffek *et al.*, 2012). In Hungary a few data on the life cycle and population dynamics of *Cacopsylla* spp. are available (Ripka, 2008; 2010; Ripka and Kiss, 2008), and only one study has been performed to survey the role of *C. pruni* in ESFY epidemiology (Süle, 2014). Although in our conditions *C. pruni* population density does usually not cause direct damages to the crops, their ability to transmit '*Ca*. P. prunorum' requires a correct orchard management in order to prevent the spread of ESFY.

The aim of our work was to determine the occurrence and population dynamics of *C. pruni* in selected apricot orchards in Hungary, to list the preferred host plants, and to gain information on the phytoplasma infection status of this psyllid species in Hungary. As no direct treatment of the disease is possible, monitoring of the psyllids provides information about the vector presence in the orchards and enables targeted control.

Recent studies in France and northern Spain demonstrated the existence of two strongly, genetically differentiated, but morphologically similar groups of *C. pruni* (Sauvion *et al.*, 2007), that often occur sympatrically. Peccoud *et al.* (2013) developed a molecular identification method to distinguish the individuals of the genetic groups A and B of the *C. pruni* species complex. This method is based on polymorphisms within the ribosomal Internal Transcribed Spacer 2 region of the two *C. pruni* genetic groups. The diagnostic PCR developed in this study by Peccoud *et al.* (2013) is a fast, cost-effective and reliable tool to assign individuals of *C. pruni* species complex to genetic groups that appear to constitute divergent species. Our objective is to classify the collected *C. pruni* specimens into the above mentioned A and B biotypes, in order to gain information about the population structure of plum psyllid in Hungary.

Materials and methods

Insects were caught from six apricot orchards in different locations in Hungary (Pest county (4), Somogy county (1), Borsod-Abaúj-Zemplén county (1) using the

combination of beating tray and sweep netting method in 2014 between beginning of March and end of June. Collections have been performed at least once in every month (altogether 4 times) at the orchards in Pest county, two times (in April and May) at Bekecs (Borsod-Abaúj-Zemplén county), and once (in April) at Somogytúr (Somogy county) (table 1). Sampling sites had been previously surveyed for the presence of '*Ca*. P. prunorum' and high ESFY disease incidence was observed in all orchards. Psyllids were collected from apricot trees and suckers, but also from wild *Prunus* species growing alongside the investigated orchards. No insecticide treatments were applied in the studied orchards.

The sample collection unit was defined as the number of psyllids obtained by beating around one tree for three minutes with a 0.28 m^2 beating tray, except for suckers, where 1-2 beat was enough. At least ten cultivated fruit and/or wild *Prunus* tree per orchard have been sampled at one collection time. The *C. pruni* falling on the tray were directly counted and collected with a mouth aspirator to confirm later their identity and sex under a stereomicroscope.

Insects were moved immediately into a sample tube containing 80% ethanol and later individually observed under stereomicroscope for proper identification and sex characterization. Samples were stored at –20 °C until tested for phytoplasma infection.

Total DNA was extracted from single psyllid individuals by modified Doyle and Doyle (1990) method. Cacopsylla DNA samples have been bulked into groups containing 10 individuals. The detection of phytoplasmas in bulked groups was performed using nested-PCR with AP group-specific primers Eof/Eor (Mergenthaler, 2004) and ESFY specific primers ECA1/ECA2 (Jarausch *et al.*, 1998). In case of having amplification product, each individual sample of the bulked groups has been tested. Amplification with Eof/Eor and ECA1/ECA2 primers was performed as follows: 3 min at 94 °C, 30 cycles of 30 sec at 94 °C, 30 sec at 55 °C, 30 sec at 72 °C and a final extension of 15 min at 72 °C. Total DNA from '*Ca*. P. prunorum' infected apricot plants were used as positive controls. PCR was performed in a final volume of 15 µL containing: 7.5 µL of ThermoScientific DreamTaq Green PCR Master Mix (2×) (containing 4 mM $MgCl_2$, 0.4 mM of each dNTP), 0.4 µM of each primer and 1 µL of DNA extract.

Molecular classification of the 281 *C. pruni* individuals into A or B genetic groups have been achieved by the ITS primer set 3 (Cp135F as universal primer and CpA425R and CpB315R as specific primers). ITS primer set 3 was chosen for amplification because this set gave the most reliable result for almost all the individuals tested by Peccoud *et al.* (2013), especially for the individuals in group B. The expected PCR fragments size are 293 bp (A) and 177 bp (B). Amplification with Cp135F, CpA425R and CpB315R primers was performed as follows: 5 min at 94 °C, 30 cycles of 30 sec at 94 °C, 20 sec at 65 °C, 30 sec at 72 °C and a final extension of 5 min at 72 °C. PCR was performed in a final volume of 15 µL containing: 1.5 µL 10× buffer, 0.625 U of *Taq* DNA polymerase, 2.5 mM $MgCl_2$, 0.4 mM of each dNTP, 0.2 µM of each primer and 1 µL of DNA extract.

PCR products were analyzed by electrophoresis through a 1% or 2% (when using the ITS primer set 3) agarose gel and visualized by staining with GelRed (Biotium, USA).

Table 1. Number of *C. pruni* collected at different sampling sites.

Location	Sampling date (2014)	Number of samples
Sóskút (Pest county)	March 26	42
	April 19	11
	May 8	16
	June 1	6
Soroksár (Pest county)	March 31	19
	April 25	13
	May 17	23
	June 04	17
Júlianna major (Adyliget) (Pest county)	March 31	39
	April 11	2
	May 24	36
	June 24	3
Paloznak (Veszprém county)	March 23	1
	April 14	6
	May 25	10
	June 12	8
Bekecs (Borsod-Abaúj-Zemplén county)	April 5	7
	May 20	11
Somogytúr (Somogy county)	April 1	12

Results

Individuals of *C. pruni* (adult reimmigrants that have overwintered and new generation) were found throughout the whole period at all localities studied. The preferred host plants appeared to be myrabolan (*P. cerasifera*), followed by blackthorn (*P. spinosa)*, European plum (*P. domestica*) and other *Prunus* species (table 2). Many of the psyllids have been collected from blackthorn and myrabolan hedges close to the orchards. Interestingly, psyllids have been found, but very rarely on apricot plants during the whole period. However, insects were caught in orchards where *P. domestica* or *P. cerasifera* were used as rootstock and provided an abundant sucker production.

The reimmigrants were found on *Prunus* from March to May and the new generation from May to June, with a peak of population at the end of April (figure 1). The migration of the new generation from host plants back onto conifers takes place at the end of June, since not any *C. pruni* was found on *Prunus* after the end of June.

The highest population level was reached in April-May, which decreased to about a half in June, when the overwintering adults already died and adults of the new generation started to move back to conifers (figure 1). Nymphs of *C. pruni* were first detected on *P. spinosa* at

Table 2. Number of *C. pruni* counted on different host plants.

Host plant	Total	Females	Males	Nymphs
P. cerasifera	108	72	27	9
P. spinosa	58	34	14	10
P. sp. (from a *Prunus* variety collection)	49	38	1	10
P. domestica	31	16	5	10
P. armeniaca suckers (e.g.: *P. cerasifera*, wild *P. armeniaca*, *P. domestica*)	19	12	7	-
Crataegus monogyna (amongst *P. spinosa*)	12	8	1	3
P. armeniaca	3	1	2	-
P. domestica suckers	1	1	-	-
Total	281	182	57	42

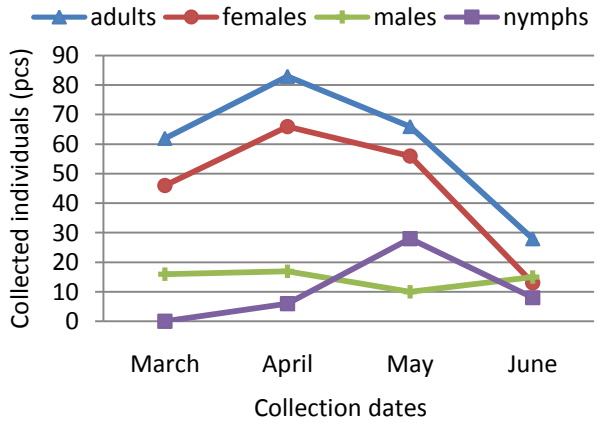

Figure 1. Presence of *C. pruni* on reproduction host plants.

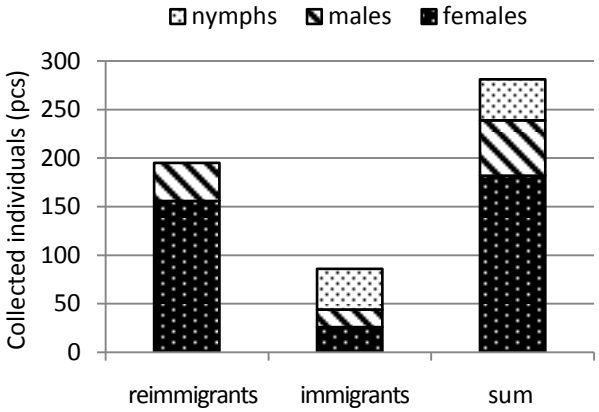

Figure 2. Composition of reimmigrant and immigrant *C. pruni* populations.

the end of April, and later from other *Prunus* species. Adults of the new generation were caught on *Prunus* in May and June.

Male and female percentages varied in the collection period, with a substantial decrease of male presence in case of reimmigrants. In contrast to this, adult males and females were close to equally represented in the new generation before they left their summer hosts for overwintering (figures 2 and 3). Results obtained on the male/female percentages when the reimmigrants were present at the orchards, evidenced the longevity of females, representing in May 85% of the population (figure 3).

The results of phytoplasma detection in the individuals of *C. pruni* captured on different host plants, in different locations and in different collection times are shown in table 3. Molecular analyses of *C. pruni* showed that 43 out of 281 tested samples were positive to '*Ca*. P. prunorum' that corresponds to 15% total infection rate. The ratio of the '*Ca*. P. prunorum' infected psyllids was uniformly 15% in the males and females, and somewhat higher (16%) in the nymphs.

Infection rates at all samplings sites were around the average 14%, except one location (Somogytúr: 8%), most probably due to the low number of samples. The higher percentage of reimmigrant individuals of *C. pruni* infected by '*Ca.* P. prunorum' was around 28% and was found in Bekecs.

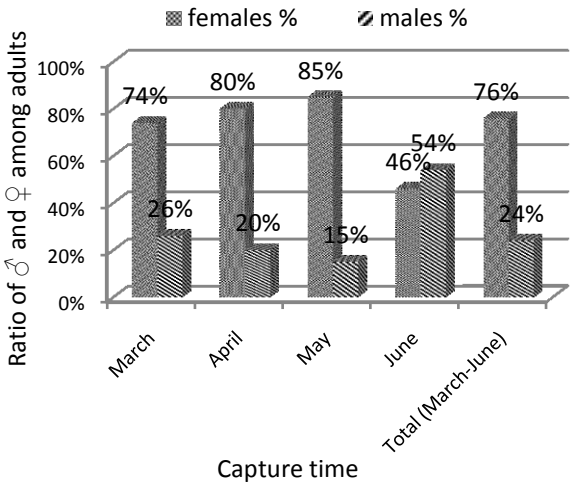

Figure 3. Ratio of males and females among adults at different collection periods.

Results also confirmed that the percentage of insects carrying the phytoplasma increased from March to June, most probably because they fed on infected trees. The infection rate of the first captured reimmigrants in March was 16%, and reached a value slightly exceeding 20% in the last captures.

'*Ca*. P. prunorum' was identified in *C. pruni* individuals collected from almost all host plants. It is remarkable

Table 3. Infection rate of *C. pruni* males, females and nymphs according to capturing times, host plants and locations (number of phytoplasma positive individuals/number of captured individuals).

	Rate of *C. pruni* individuals assigned to be '*Ca*. P. prunorum' positive			
	% total	% females	% males	% nymphs
Total collection period	43/281 (15%)	27/181 (15%)	9/58 (15%)	7/42 (16%)
Capture periods				
March	10/62 (16%)	6/46 (13%)	4/16 (25%)	-
April	8/89 (9%)	5/66 (8%)	3/17 (18%)	0/6 (0%)
May	17/94 (18%)	14/56 (25%)	1/10 (10%)	2/28 (7%)
June	8/36 (22%)	2/13 (15%)	1/15 (7%)	5/8 (62%)
Host plants				
P. cerasifera	18/108 (17%)	8/72 (11%)	5/27 (19%)	5/9 (55%)
P. spinosa	12/58 (21%)	8/34 (24%)	2/14 (14%)	2/10 (20%)
other *P.* sp.	6/49 (12%)	6/38 (16%)	0/1 (0%)	0/10 (0%)
P. domestica	3/31 (10%)	3/16 (19%)	0/5 (0%)	0/10 (0%)
P. armeniaca suckers	3/19 (16%)	1/12 (8%)	2/7 (28%)	-
Crataegus monogyna	0/12 (0%)	0/8 (0%)	0/1 (0%)	0/3 (0%)
P. armeniaca	0/3 (0%)	0/1 (0%)	0/2 (0%)	-
P. domestica suckers	1/3 (33%)	1/3 (33%)	-	-
Sampling sites				
Julianna-major	14/77 (18%)	5/44 (11%)	4/25 (16%)	5/8 (62%)
Sóskút	12/77 (16%)	6/51 (12%)	4/20 (20%)	2/6 (33%)
Soroksár	9/72 (13%)	9/48 (19%)	0/5 (0%)	0/19 (0%)
Paloznak	3/24 (12%)	3/11 (27%)	0/4 (0%)	0/9 (0%)
Bekecs	5/18 (28%)	4/17 (24%)	1/1 (100%)	-
Somogytúr	1/13 (8%)	1/10 (10%)	0/3 (0%)	-

that the ratio of infected insects from *P. spinosa* and suckers are slightly above the average, which suggests that rootstocks and blackthorn are preferred feeding site for *C. pruni* and those can be considered as an important inoculum source.

Molecular characterization of *C. pruni* individuals performed with the ITS primer set 3 showed the presence of a 177 bp amplified fragment in all tested groups of individuals (figure 4B), corresponding to the genetic group B. None of the 29 groups produced amplicons of different sizes, so all groups can be considered homogenous. The collected *Cacopsylla crataegi* (Schrank) and *Cacopsylla picta* (Foerster) individuals identified on the base of morphological traits produced no PCR amplicons (figure 4A).

Discussion

This survey confirmed the occurrence of *C. pruni* and its moderate infectivity in the sampled regions, where ESFY disease is endemic and affects both cultivated and wild *Prunus* species. This infectivity rate is in agreement with records reported from other countries in Europe. In France, the highest range of phytoplasma infected reimmigrant *C. pruni* was recorded as 15% but is generally around 3% (Yvon *et al*., 2004). In Turkey, this range was determined as 23% what seems somewhat higher (Serçe *et al.*, 2011). Ermacora *et al*. (2011) determined in a two year survey in North-Eastern Italy, that the infection rate of the first capture reimmigrants was 56.4% and reached a plateau slightly exceeding 80%.

Concerning phytoplasma infection of the species captured in the studied orchards, our results showed that the overwintering as well as in the new generation of *C. pruni* carried the phytoplasma. It means that *C. pruni* can act as a vector of '*Ca*. P. prunorum' both as overwintered and springtime adults. These findings fit with other studies that both generations are infected (Fialova *et al.*, 2007), however some authors ascribe higher significance to the overwintering generation in phytoplasma transmission (Thébaud *et al.*, 2008).

Since not only sparse, but prevalent incidence of immigrants have been found on different *Prunus* species from the middle of March, the migration of psyllids from conifers onto host plants could have been started already at the beginning of March or even at the end of February. Immatures of *C. pruni* were first detected on *P. spinosa* at the end of April, and later from other *Prunus* species. This is in agreement with the observations of Labonne and Lichou (2004), those who designated blackthorn as a "sentinel species". The first activity of the insect could be detected more easily on *P. spinosa* than in orchards on cultivated *Prunus* species.

P. cerasifera and *P. spinosa* appeared to be two most preferred host plants in Hungary compared to the other *Prunus* spp. studied. This host plant preference is partly in agreement with previous records from other countries (Labonne and Lichou, 2004, Serçe *et al.*, 2011).

Experiencing the presence of *C. pruni* individuals on suckers lead to the instant removal of these shoots in selected apricot orchards. This is the reason for data showing the occurrence only of adults of *C. pruni* and not of nymphs on suckers.

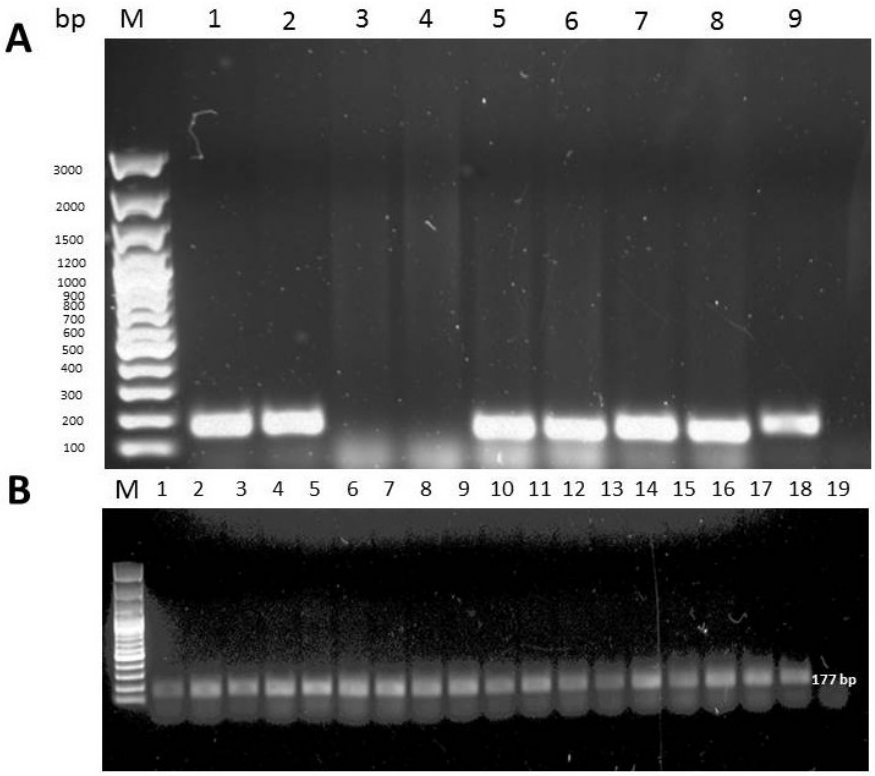

Figure 4. Amplicons from PCRs using the ITS primer set 3 (Cp135F, CCpB315R, CpA425R) for the classification of *C. pruni* individuals into A or B genetic groups. M: Generuler 100 bp Plus DNA Ladder (Fermentas). **A)** lanes 1-2: *C. pruni*, lane 3: *C. picta*, lane 4: *C. crataegi*, lanes 5-8: *C. pruni*, lane 9: *C. pruni* (30-fold dilution of the DNA sample used in lane 6); **B)** lanes 1-18: bulked groups representing *C. pruni* DNA samples from 1 to 180, lane 19: negative control.

Surprisingly, we were able to find only very few individuals of *C. pruni* on apricot trees during the whole period. This raises the question of whether only that insect species alone is responsible for the quick spreading of this lethal disease in Hungarian apricot orchards. This has to be answered in the future, nevertheless the key role of the uncontrolled phytoplasma-infected propagation material is suggested.

Results obtained on the male/female percentages when the reimmigrant individuals are present in the orchards, evidenced the longevity of females that at the end of the monitored period represented 88.5% of the population. The longevity of females could be explained from an evolutionary point of view considering the crucial role of the females for the species survival.

The diagnostic PCR developed by Peccoud *et al.* (2013) proved to be a fast and reliable tool to assign individuals of *C. pruni* to genetic groups. All the collected insects could be unambiguously defined into the genetic group B. Our results are congruent with the observations of this study, where samples originated from the eastern part of Europe (Serbia, Czech Republic, Northern Italy, Germany) and from Asia (Turkey) contained individuals uniformly classified into genetic group B. A recent study (Oettl and Schlink, 2015) refine further the problem of *C. pruni* species complex, indicating the presence of not only A and B, but further genetic groups of *C. pruni*. These and other studies support the necessity of further analysis on *C. pruni* genetic groups. Several authors hypothesized putative connection between host plant preference/transmission efficiency and genetic variants of different *Cacopsylla* species (Sauvion *et al.*, 2007, Tedeschi and Nardi, 2010). These molecular techniques could be a useful tool for study on distribution of plum psyllid, its host plants and its ability of the '*Ca.* P. prunorum' transmission.

In conclusion, occurrence of the plum psyllid in Hungary have been confirmed both with morphological and molecular methods. Our studies show the presence of the vector belonging to genetic group B in all the investigated localities and the high infection rates reveal a high spread risk of '*Ca.* P. prunorum' by *C. pruni* in Hungary. Future investigations will aim to monitor regularly *C. pruni* in these and other fruit tree growing areas of Hungary for possible phytoplasma infections.

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