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MOLECULAR TYPING OF BULGARIAN SPECIMEN OF THE PHYTOPLASMA VECTORS *CACOPSYLLA PRUNI* SCOPOLI AND *CACOPSYLLA MELANONEURA* (FOERSTER)

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Abstract

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European fruit tree phytoplasmas diseases apple proliferation, pear decline and European stone fruit yellows are among the most important and rapidly spreading diseases in Bulgaria. Their causal agents '*Ca.* Phytoplasma mali', '*Ca.* Phytoplasma pyri' and '*Ca.* Phytoplasma prunorum' and their known and putative vectors have been identified in Bulgaria recently. This is the first report about the molecular characterisation of the phytoplasma vector species *Cacopsylla pruni* Scopoli and *Cacopsylla melanoneura* (Foerster) in Bulgaria. The results confirmed the presence of *Cacopsylla pruni* type B in all Bulgarian samples and demonstrated a genetic variability among *C. melanoneura* specimen. For the first time, genetic variants WI and WOI were described outside Italy, but a relative high number of *C. melanoneura* could not be characterised by this molecular typing approach. Molecular identification of *Cacopsylla affinis*, a potential vector of different fruit tree phytoplasmas, proofed to be reliable.

Key words: apple proliferation, European stone fruit yellows, '*Candidatus* Phytoplasma mali', '*Candidatus* Phytoplasma prunorum', *Cacopsylla affinis*

Introduction

In the past years, phytoplasma diseases are one of the most important threats of fruit trees in Bulgaria and until now the existence and spread of fruit tree phytoplasmas and their vectors have not been studied in detail. In our previous work (Etropolska et al., 2011; Etropolska and Laginova, 2012; Etropolska et al., 2015) all three European fruit tree phytoplasmas '*Ca.* Phytoplasma mali', the agent of apple proliferation, '*Ca.* Phytoplasma pyri', the agent of pear decline and '*Ca.* Phytoplasma prunorum', the agent of European stone fruit yellows (Seemüller and Schneider, 2004) were found to be present in Bulgaria. All known vectors of these fruit tree phytoplasmas were also found in Bulgaria: *Cacopsylla picta* and *Cacopsylla melanoneura*, the vectors of '*Ca.* P. mali', *Cacopsylla pyri* and *Cacopsylla pyricola*, the vectors of '*Ca.* P. pyri' and *Cacopsylla pruni*, the vector of '*Ca.* P. prunorum', reviewed in Jarausch and Jarausch (2010). In addition, putative vectors like *Cacopsylla pyrisuga, Cacopsylla bidens* or *Cacopsylla affinis* were identified (Etropolska et al., 2015).

However, there are increasing data which demonstrate that different populations of these psyllid vectors exist which might have different phytoplasma transmission capacities and, thus, represent different levels of risk for the disease spread. A striking example is *C. melanoneura* which has been described as only vector of '*Ca.* P. mali' in Northwestern Italy (Tedeschi et al., 2002; Tedeschi and Alma, 2004) but which is no vector of the disease in Germany (Mayer et al., 2009). In addition, this species can be found on two main plant hosts: *Crataegus* (hawthorn) and *Malus* (apple). Recent work of Malagnini et al. (2013) demonstrated the existence of ecologically and genetically different populations of *C.*

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melanoneura using microsatellite markers. These authors could distinguish populations from *Crataegus* and *Malus* while in other work *C. melanoneura* collected from hawthorn was found to carry '*Ca.* P. mali' and '*Ca.* P. pyri' (Tedeschi et al., 2009; Tedeschi and Nardi, 2010). Also in Bulgaria, we found *C. melanoneura* infected with '*Ca.* P. pyri' (Etropolska et al., 2015). The presence of a genetic variability among *C. melanoneura* was already observed by Tedeschi and Nardi (2010) who identified two different genetic profiles (called WI and WOI) in the mitochondrial control region of Italian populations collected from apple and from hawthorn, respectively. However, no morphological differences between the populations were detected.

The species *C. melanoneura* is regularly found in mixed populations with other *Cacopsylla* species: with the main '*Ca.* P. mali' vector *C. picta* on *Malus* and with *C. affinis* - mainly on *Crataegus,* but sometimes also on *Malus.* All three species are morphologically very similar, have a comparable biology and can be found at the same time in the orchards during the vegetation. It is very difficult to determinate them morphologically, especially by a non-specialist. In addition, Tedeschi at al. (2009) and Tedeschi and Nardi (2010) reported that individuals of *C. affinis* can also carry '*Ca.* P. mali', but their vector capacity was not confirmed in transmission trials. Therefore, correct species identification is necessary to clarify the role of each psyllid species in the spread of apple proliferation in Europe and in Bulgaria, respectively. Tedeschi and Nardi (2010) developed a molecular tool to distinguish between *C. melanoneura* and *C. affinis*.

Genetic variability was also described for another important phytoplasma vector: *Cacopsylla pruni*. Sauvion et al. (2007) studied in detail the population structure of *C. pruni* from 12 different locations in southern France and in northern Spain and identified two differentiated populations of *C. pruni* spp, called type A and type B. In further work, Peccoud et al. (2013) hypothesised that these two types might even be two different species. However, both types are able to transmit '*Ca.* P. prunorum' but their individual risk for the disease spread is still unknown. Based on sequence data of the internal transcribed spacer 2 Peccoud et al. (2013) developed a diagnostic molecular test to distinguish between *C. pruni* type A and B. The geographic distribution of these two types in Europe is still largely unknown. As Bulgaria geographically bridges Central Europe with Asia, the identification of the existing genotypes of *C. pruni* in this region is of particular interest for the understanding of the ESFY-disease spread not only in Bulgaria but as well as across Europe.

The aim of this work was the molecular characterisation of the main vector of '*Ca.* P. prunorum' and known and putative vectors of '*Ca.* P. mali' in Bulgaria. For this, available molecular markers and protocols (Tedeschi and Nardi, 2010; Peccoud et al., 2013) were applied.

Materials and Methods

Morphological identification of psyllids

Insects were caught using sweep netting during the period March-July of each year in 2011-2013 as described in Etropolska et al. (2015). Captured psyllids were frozen at -20°C and species identification was done using the determination keys of Hodkinson and White (1979), Ossiannilsson (1992) and the electronic key of Burckhardt and Jarausch (2007).

Total DNA extraction of insects

DNA was extracted from each psyllid individual with the modified CTAB-based protocol according to Jarausch et al. (2011) or with the TNES protocol, presented by Nicolas Sauvion at the COST FA0807 molecular identification training school in Montpellier, September 2012 (Sauvion, N., 2012).

Molecular typing of C. pruni

Molecular typing of *C. pruni* specimen was done according to the protocol of Peccoud et al. (2013): the universal primer Cp480R (5'-TACATCCGAGGGTCGGTATC-3') was used in triplex PCR together with the group-A specific primer CpA300F (5'- GGCCAGTAGTTAAACCGGACT-3') and the group-B specific primer CpB120F (3'-TCCACGGGGTCCGCGATA-5'). The PCR was performed in a final volume of 20µl with 1x Taq polymerase buffer with 2.5 mM $MgCl_2$, 0.125 mM of each dNTP, 0.25 µM of each primer, 0.5 U of 5prime Taq polymerase (5prime, Germany) and 1 µl of total insect DNA. PCR cycles were as follows: initial denaturation at 95°C for 1 min; followed by 35 cycles at 95°C for 15 sec, 65°C for 20 sec and 72°C for 30 sec, with a final 4-min extension period at 72°C. PCR products were analysed on 2% agarose gel stained with ethidium bromide for visualization under UV light. Specific PCR products of *C. pruni* type A had a size of 172 bp, whereas PCR products of *C. pruni* type B had a size of 377 bp. Positive controls for *C. pruni* types were kindly provided by Nicolas Sauvion (INRA Montpellier).

Molecular typing of C. melanoneura

For molecular typing of *C. melanoneura* specimen the PCR discrimination system developed by Tedeschi and Nardi (2010) was employed. Primer pair MEL_fw (5'-TTTTATCCACTCTTAAAGCTTG-3') and MEL_rev (5'-TGATAGAGCTTTTTGAATTCTC-3') allows a specific detection of *C. melanoneura* specimen. Furthermore, this primer pair can discriminate between two genetic variants of *C. melanoneura*: type WOI yields a 436 bp PCR product while type WI

gives rise to the amplification of a 381 bp PCR product. Primer pair AFF_fw (5'-TTTAACCACCTCAAACTCAA-3') and AFF_rev (5'-CGTAAAATTCTTGGCGA-3') specifically amplifies a 499 bp PCR product from *C. affinis* specimen. Both primer pairs can be used with the same PCR reaction and cycling conditions. In this study the PCR reaction was set up in a final volume of 20µl with 1x Taq polymerase buffer with 1.5 mM $MgCl_2$, 0.125 mM of each dNTP, 1 µM of each primer, 0.5 U of 5prime Taq polymerase (5prime, Germany) and 1 µl of total insect DNA. PCR cycles were as follows: initial denaturation at 95°C for 1 min; followed by 40 cycles at 95°C for 15 sec, 55°C for 20 sec and 72°C for 45 sec, with a final 4-min extension period at 72°C. PCR products were analysed on 2% agarose gel stained with ethidium bromide for visualization under UV light.

Results and Discussion

Molecular characterisation of Cacopsylla pruni

In total, 565 *Cacopsylla pruni* individuals from four different sites in Bulgaria were captured and identified morphologically. Infection with '*Ca.* Phytoplasma prunorum' was confirmed in 13 individuals at all four sites (Etropolska et al., 2015). A representative number of 437 *C. pruni* individuals was genetically analysed according to the typing system published by Peccoud et al. (2013). Using this triplex PCR approach all Bulgarian *C. pruni* samples yielded a PCR product of 377 bp (Figure 1). As summarised in Table 1, all tested *C. pruni* specimen of Bulgaria were of type B.

This result confirmed fragmentary data of Peccoud et al. (2013) who found *C. pruni* type B in one site in Serbia as well as in Turkey. *C. pruni* type B is also widespread in Germany (Jarausch et al., 2014). So far, *C. pruni* type A was only detected in France and northern Spain. *C. pruni* type B is regarded as the "true" *C. pruni* type. Thus, according to our data, "true" *C. pruni* is present in Bulgaria. The '*Ca.* P. prunorum' infection rates of *C. pruni* type B in Germany - 2% according to Jarausch et al. (2007a,b) and in Bulgaria - 2.3% according to Etropolska et al. (2015) are almost identical indicating that the *C. pruni* populations are similar and, thus, the risk for ESFY disease spread is comparable. Our results further support the hypothesis that *C. pruni* type B is the dominant or only type in Middle and Eastern Europe.

Molecular characterisation of Cacopsylla melanoneura

At the same period psyllids were collected from *Malus* as well as from *Crataegus* plants at different locations in Bulgaria. After the morphological identification we found mixed populations of *C. picta* and *C. melanoneura* on *Malus* and mixed populations of *C. melanoneura*, *C. affinis* and *C.*

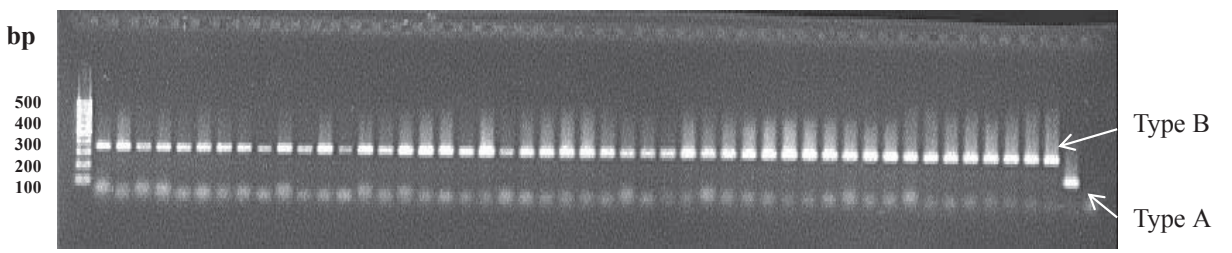

Fig. 1. 2% Agarose gel electrophoresis of triplex PCR products amplified from Bulgarian *C. pruni* individuals. Lanes 1 to 46: Bulgarian *C. pruni* samples, lane 47: *C. pruni* type B reference sample (377 bp), and line 48: *C. pruni* type A reference sample (172 bp)

Table 1
Molecular typing of *C. pruni* individuals from different sites of Bulgaria

Site	Years	Host plant	Number of individuals	Typing	Size of PCR product
Kjustendil/Dupnica	2011, 2012, 2013	P. domestica	179	B	377 bp
Sofia/Lozen	2011, 2013	P. domestica	209	B	377 bp
Sofia/Pancharevo	2012	P. domestica	11	B	377 bp
Kjustendil/Jabulkovo	2013	P. domestica	21	B	377 bp
Kjustendil City	2013	P. domestica	17	B	377 bp
Total			**437**		

crataegi on *Crataegus* spp. The morphological identification of female individuals is particularly difficult, especially between *C. melanoneura* and *C. affinis*. Therefore, the molecular tools developed by Tedeschi and Nardi et al. (2010) for identification of either *C. melanoneura* or *C. affinis* were evaluated. Among 280 individuals captured in Bulgaria, 233 insects were morphologically identified as *C. melanoneura* and 47 individuals were morphologically identified as *C. affinis*. Total DNA was extracted from all specimens and subjected to PCR with either primer pair MEL_fw/MEL_rev for identification of *C. melanoneura* or primer pair AFF_fw/ AFF_rev for identification of *C. affinis*. The results in Table 2 show that *C. melanoneura* was confirmed in 129 samples. All 78 negative samples were tested with AFF_fw/ AFF_rev and *C. affinis* was confirmed in 37 out of them. Most of the morphologically misidentified *C. melanoneura* were female individuals which are very difficult to distinguish from female individuals of *C. affinis*. However, 41 samples did not react with either primer pair. Compared to this, *C. affinis* identification was more reliable as only 2 individuals were misidentified and another 2 samples did not react with any primer pair. All samples which reacted negatively with both primer pairs contained amplifiable DNA as tested with a universal *Cacopsylla* detection primer pair (Jarausch et al., 2010) (data not shown). As both primer pairs were already successfully applied by Malagnini et al. (2013) for differentiation of *C. melanoneura* and *C. affinis* in Northern Italy, the question remains if *C. melanoneura* populations in Bulgaria are different. Further analyses are needed to clarify this situation.

The molecular analysis of *C. melanoneura* samples of Bulgaria with primer pair MEL_fw/MEL_rev enabled also the identification of genetic variability of *C. melanoneura* specimen. As shown in Figure 2, for the first time genetic variant WI could be detected in one individual outside Italy. However, 128 out of the 129 analysed individuals were of WOI type. Although the WI type is always in minority, Tedeschi and Nardi (2010) reported much higher frequencies of WI type – ranging from 5.0 – 31.3% - in different geographic regions of Italy. They also devised the hypothesis, that a higher percentage of WOI type is correlated with a higher infection rate of *C. melanoneura* with '*Ca.* P. mali' and, thus, with a higher risk of AP disease spread by *C. melanoneura*. In this regard, more work needs to be done to clarify the epidemiological role of *C. melanoneura* for AP spread in Bulgaria. So far, *C. melanoneura* has not been found infected with '*Ca.* P. mali' in Bulgaria (Etropolska et al., 2015) while *C. picta*, the main vector of '*Ca.* P. mali', reached an infection rate of 2.8%.

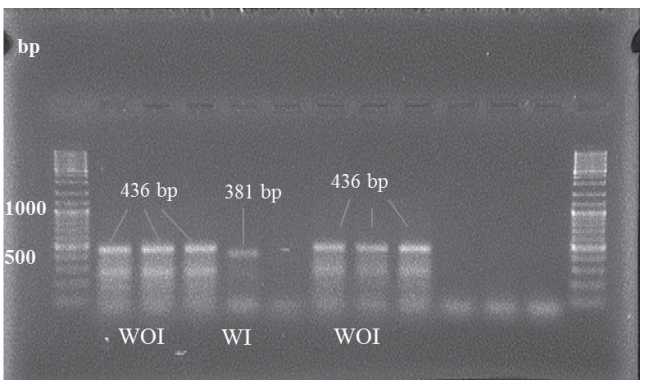

Fig. 2. Identification of genetic variants WI and WOI among Bulgarian *C. melanoneura* individuals. 2% Agarose gel electrophoresis of MEL_fw/MEL_rev PCR products: lanes 1 to 3 and 6 to 8 – *C. melanoneura* WOI; lane 4 – *C. melanoneura* WI.

Conclusion

In conclusion, a first molecular characterisation of *Cacopsylla pruni* and *Cacopsylla melanoneura* in Bulgaria was carried out. Our results for *C. pruni* indicate that the Bulgarian population is similar to the Middle and Eastern European populations and is supposed to have the same potential to transmit '*Ca.* P. prunorum' also in Bulgaria. Regarding the results of the molecular characterisation of *C. melanoneura* we can confirm that the two genotypes of *C. melanoneura* exist in Bulgaria. However, the apparent dominance of the WOI type as well as the high number of uncharacterised *C. melanoneura* specimen reveal the need for further studies on *C. melanoneura* populations in Bulgaria in order to clarify the role of this species in the spread of apple proliferation in Bulgaria.

Table 2
Results of the morphological and molecular determination of *C. melanoneura* and *C. affinis* specimen of Bulgaria

Psyllid species	Morphological identification	PCR test positive / total		Negative with both primer pairs / total
		MEL_fw/ MELrev	AFF_fw/ AFF_rev	
C. melanoneura	233	129 / 233	37 / 233	41 / 233
C. affinis	47	2/47	43 / 47	2/47

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