COST Action FA0807 Integrated Management of Phytoplasma Epidemics in Different Crop Systems
Short-term Scientific Mission (STSM) Report

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STSM Topic: Molecular characterization of 16SrX group phytoplasma vectors from Bulgaria

Purpose of the visit:

The aim of the visit at the AIPlanta institute was the molecular characterization of all known and putative vectors of the fruit tree phytoplasmas in Bulgaria with the available molecular tools. The work was based on available marker genes and protocols (Tedeschi & Nardi, 2010; Sauvion et al., 2007 and Peccoud et al., 2013) as well as on the analysis of new genome markers or endosymbiont markers as proposed by Thao et al. (2000). Also, new protocols presented at the molecular identification training school at Montpellier, November 2012, during the COST WG2 activities were tested.

Background:

In recent years, phytoplasma diseases are one of the most important diseases 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. As Bulgaria geographically bridges Central Europe with Asia, the molecular characterisation of 16SrX phytoplasma strains and their vectors in this region is of particular interest for the understanding of the disease spreading in Europe.

Support of COST action FA0807 has already enabled in a previous STSM to characterize 16SrX group phytoplasma isolates from Bulgaria. All three European fruit tree phytoplasmas, '*Ca.* Phytoplasma mali', '*Ca.* Phytoplasma pyri' and '*Ca.* Phytoplasma prunorum', are present in Bulgaria. In previous work, all acknowledged vectors of the fruit tree phytoplasmas have been found in Bulgaria: *Cacopsylla picta*, *Cacopsylla melanoneura*, *Cacopsylla pyri*, *Cacopsylla pyricola,* and *Cacopsylla pruni*, and also putative vectors like *Cacopsylla pyrisuga, Cacopsylla bidens* or *Cacopsylla affinis.* Recent work has shown, however, that different populations with different transmission capacities might exist for a given vector species. E.g., *C. melanoneura* is a vector in Northern Italy but not in Germany (Mayer et al., 2009); genetic differences among populations of *C. melanoneura* have been reported (Tedeschi & Nardi, 2010). *C. pruni* is going to be divided into two distinct species (Sauvion et al., 2007; Peccoud et al., 2013). On the other hand, different psyllid species with similar morphology may co-exist on the same host plant and are sometimes difficult to

distinguish. One example is mixed populations of *C. melanoneura* and *C. affinis* which seem to be very frequent in Bulgaria, another problem are mixed populations of *Cacopsylla pyri*, *Cacopsylla pyricola, Cacopsylla pyrisuga* and *Cacopsylla bidens* which have been found together in the pear orchards in Bulgaria. Thus, molecular tools for species identification or genetic differentiation of populations are needed. Here, the hosting laboratory in Neustadt has great experience (Jarausch et al., 2010) and can provide a large collection of DNA from different psyllid species or populations.

Results and discussion:

Morphological identification and DNA extraction

Captured psyllids were frozen at -20°C and psyllid species identification was done in AIPlanta laboratory and also with the kind support from Dr. Daniel Burckardt, Naturhistorisches Museum, Basel, using different determination keys (Ossiannilsson, 1992; Burckhardt et al., 2008). DNA was extracted from more than 400 psyllid individuals with the modified CTAB-based protocol according to Jarausch *et al.* (2011) and from more than 200 individuals with the TNES protocol, presented by Nicolas Sauvion at the COST FA0807 molecular identification training school in Montpellier, November 2012.

All *Cacopsylla* individuals were tested with universal ribosomal primer fU5/P7 (Lorenz *et al.*, 1995; Schneider *et al.*, 1995), and with primer pair WG1/2 (Jarausch et al., 2010). '*Ca.* P. mali' was identified also with specific primers AP3/AP4 (Jarausch *et al.*, 1994) and '*Ca.* P. prunorum' by non-ribosomal primers ECA1/ ECA2 (Jarausch *et al.*, 1998). For '*Ca.* P. pyri' identification the PD-specific primer pair fPD/rPD (Etropolska *et al.*, 2011) was used. PCR amplification was performed in 20 µl reaction volume as described in the respective publication.

Table 1 Number of tested insect samples collected in different regions of Bulgaria from 2011 until 2013.

Insect spp.	Host plants	Samples	Phytoplasma positive	Phytoplasma type	Region
C. picta	*M. domestica*	70	2	'*Ca.* P. mali'	Sofia, Kjustendil
C. mali	*M. domestica*	3	0		Sofia, Kjustendil
C. pruni	*P. domestica*	437	9	'*Ca.* P. prunorum'	Sofia, Kjustendil
C. melanoneura	*M. domestica*	132	0		Sofia, Kjustendil
C. melanoneura	*Crataegus*	93	1	'*Ca.* P. pyri'	Sofia, Kjustendil
C. affinis	*Crataegus*	62	0		Kjustendil
C. pyri	*P. communis*	296	2	'*Ca.* P. pyri'	Sofia, Kjustendil, Petrich
C. pyricola	*P. communis*	88	2	'*Ca.* P. pyri'	Sofia, Kjustendil
C. pyrisuga	*P. communis*	322	0		Sofia, Kjustendil
C. bidens	*P. communis*	29	1	'*Ca.* P. pyri'	Sofia, Kjustendil

For this work we used more then 1500 already extracted DNA isolates, from different psyllid species, caught from 2011 to 2013 on different host plants and from different regions in Bulgaria and also DNA isolate of other insects from AIPlanta's DNA collection.
Different specific primers were used or newly designed for the molecular characterisation of the psyllid species.

Cloning, sequencing, sequence analysis

For the sequence analysis of different molecular markers 1 µl of the respective PCR product was ligated into pTPCR vector (Wassenegger unpublished) and cloned in competent *Echerichia coli* Invα cells. DNA of recombinant plasmids was extracted with a QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instruction. Cloned fragments were sent for sequencing.

The analysis of the sequences was done by DNAstar Lasergene software program and for the aligments the ClustalW algorithm was applied.

Molecular characterisation of *Cacopsylla pruni*:

The molecular characterisation of *Cacopsylla pruni* was done through molecular typing (Triplex method) according to Peccoud et al. (2013). In the publication of 2007 Sauvion et al. studied the population structure of *Cacopsylla 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. For the molecular characterisation of *C. pruni* in Bulgaria we applied this typing method to all *C. pruni* samples from different locations in Bulgaria and Germany.

For the molecular typing PCR amplification was performed with the primer combination CpA300F + CpB120F + Cp480R in 20 µl reactions with 0,25µM of each primer. The PCR amplification parameters were 35 cycles 15 sec at 95 ^{0}C, 20 sec at 65 ^{0}C, 30 sec at 72 ^{0}C. The PCR products were separated on a 2 % agarose gel and visualized through ethidium bromide under UV light.

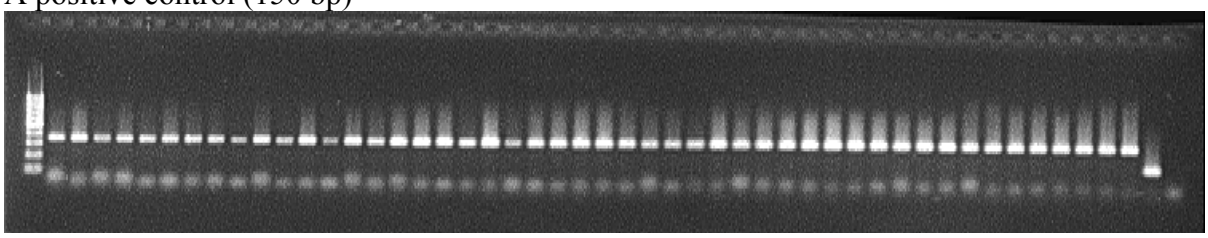
The recognized *C. pruni* – Type- A had 150 bp, and *C. pruni* – Type - B had 350 bp. For positive controls we used a *C. pruni* Type- A isolate kindly provided by N. Sauvion.
In total 437 *C. pruni* individuals were tested with the triplex method. The samples were collected in several sites in Sofia - and Kjustendil Districts, during the years 2011- 2013.

Table 1. Results of all tested *C. pruni* individuals with the molecular typing.

Site	Years	Host plant	Number of individuals	Typing	bp
Kjustendil/Dupnica	2011, 2012, 2013	P. domestica	179	**B**	**350**
Sofia/Lozen	2011, 2013	P. domestica	209	**B**	**350**
Sofia/Pancharevo	2012	P. domestica	11	**B**	**350**
Kjustendil/Jabulkovo	2013	P. domestica	21	**B**	**350**
Kjustendil City	2013	P. domestica	17	**B**	**350**
Total			**437**		

The results showed that all tested *Cacopsylla pruni* individuals were of type B.

Figure 1. 2 % Agarose gel electrophoresis of triplex PCR products amplified from Bulgarian *C. pruni* samples. Lines 1 to 47: *C. pruni* – Type - B (350 bp), and line 48: *C. pruni* – Type- A positive control (150 bp)



Molecular characterisation of *Cacopsylla melanoneura*:

During the last 3 years psyllids were collected from *Malus* - and *Crataegus* plants from different locations in Bulgaria. After the morphological identification we found mixed populations from *C. picta* and *C. melanoneura* on *Malus* and mixed populations of *C. melanoneura*, *C. affinis* and *C. crataegi* on *Crategus* spp. Particularly the morphological identification of female individuals is very difficult, especially differentiation between, *C. melanoneura* and *C. affinis*, and therefore, supported by the experience of Tedeschi & Nardi et al. (2010) we wanted to compare the morphological and the molecular tools for psyllid species identification. We tested all morphologically described individual as *C. melanoneura* or as *C. affinis* from Bulgaria with the specific mitochondrial primer pair MEL_fw/MEL_rev and primer pair AFF_fw/AFF_rev developed by Tedeschi & Nardi et al. (2010). It was also very interesting to check, if in the Bulgarian *C. melanoneura* populations genetic variants could be identified which were named by Tedeschi & Nardi et al. (2010) *C. melanoneura* WI and *C. melanoneura* WOI.

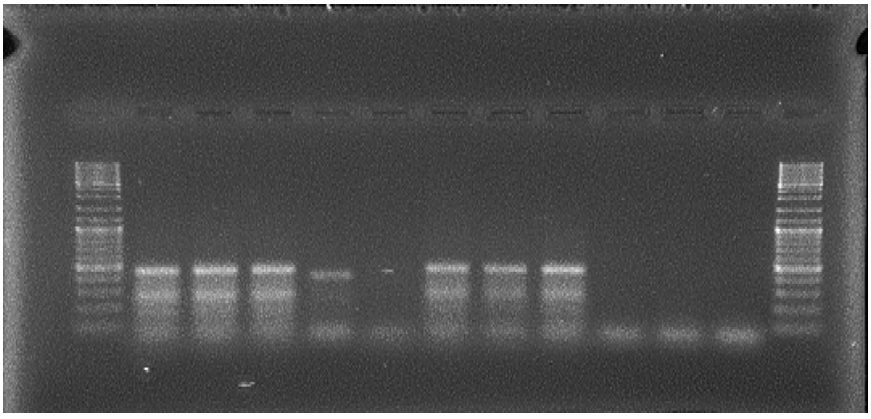
In total DNA was extracted with the CTAB based protocol from 280 individuals, 233 insects were morphologically identified as *C. melanoneura* and 47 individuals were morphologically identified as *C. affinis*. The results (Table 2) of the specific primer test showed, that *C. melanoneura* was confirmed 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*. A high number of samples (41) did not react with either primer pair and could not be identified by molecular means. Further tests will be applied to these samples.

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

Psyllid species	Morphological identification	PCR positive / total tested		Negative with both primer pairs
		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

Regarding to the publication of Tedeschi & Nardi et al. (2010) about the two genetic variants of *C. melanoneura*, named as WI (381 bp) and WOI (436 bp), our results showed, that from all 129 individuals 128 were identified as WOI and only 1 individual was WI (Figure 2)

Figure 2. Genetic variants of *C. melanoneura.* Lines 1 to 3 and 6 to 8 – *C. melanoneura* WOI; line 4 – *C. melanoneura* WI.



Molecular characterisation of *Cacopslla pyri, C. pyricola and C. pyrisuga:*

During the last 3 years psyllids were collected also in pear orchards in different locations. After the morphological identification in the AIPlanta laboratory and at the Naturhistorisches Museum, Basel, four *Cacopsylla* species were identified: *Cacopsylla pyri* the main vector of '*C*a.Phytoplasma pyri' was found in one pear orchard, infected with '*C*a.'Phytoplasma pyri'. Two individuals were also infected. In all other locations on the territory of Sofia and Kjustendil districts mixed populations of *C. pyricola* and *C. pyrisuga* were found. In samples from this year we found also individuals of *C. bidens*. All individuals were tested for phytoplasma infection. In 2 individuals of *C. pyricola* and 1 individual of *C. bidens* '*Ca.* P. pyri' was detected. With respect to this results and the fact, that pear decline is the most spread phytoplasma disease in Bulgaria (Etropolska A, and Laginova M, 2012), the main question which is the vector/vectors of '*C*a. P. pyri' in Bulgaria is still to be answered. This work is hampered by the difficulties to identify and distinguish morphologically the four *Cacopsylla* species found in the pear orchards – in particular the females. Therefore, our work was focused to identify genetic differences between *C. pyri* and other pear psyllids in order to develop molecular tools for vector identification because no molecular assays are available for now. In order to develop specific primers for the identification of *Cacopslla pyri, C. pyricola and C.* pyrisuga we analysed the obligate psyllid endosymbiont *Carsonella rudii* which strictly co-evolve with the psyllid species (Thao et al., 2000). We sequenced fragments of the endosymbiont 16SrDNA and developed specific primers in variable regions of the 16SrDNA. The first results which could be obtained during this STSM demonstrated the specificity of the primers for the identification of *C. pyri*, *C. pyricola* and *C. pyrisuga.* However, further validation is needed.

Future collaboration

The collaboration between both institutes will therefore be continued to further elucidate the phylogeographic situation of Bulgarian psyllid populations and to understand the past and recent spread of fruit tree phytoplasmas and there vectors in this country. In future the results of the both STMS's will be very helpful for my PhD work.

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