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 isolates from Bulgaria

Purpose of the visit

The aim of the visit at the AIPlanta institute was the molecular characterization of 16SrX group phytoplasma isolates from Bulgaria with the available molecular tools and the comparison of the data with published data from other geographic regions.

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 in this region is of particular interest for the understanding of the disease spreading in Europe. Therefore it is very interesting to analyze, if there are differences between the Bulgarian isolates and the known European isolates and also if there is diversity of phytoplasmas in the same infected tree, particularly by '*Ca*. Phytoplasma mali'. As basic information we used the molecular typing systems published by Danet *et al.* (2011) and Seemüller *et al.* (2010; 2011) by sequencing fragments of aceF, pnp, imp and hflB genes.

Material & Methods:

Phytoplasma isolates were obtained from *Malus*, *Pyrus* and *Prunus* trees in different regions of Bulgaria as well as from phytoplasma-infected insects. During the STSM available DNA extracts of infected samples from 2010 and 2011 were used as well as new insect and plant samples from symptomatic trees collected in the growing season 2012. A summary is given in Tab. 1.

Table 1 Samples from plant and insect collected in different regions of Bulgaria

Plant/Insect spp.	Host plants	Samples	Region
M. domestica		29	Plovdiv, Sofia, Kjustendil, Petrich
P. domestica		27	Plovdiv, Sofia, Kjustendil
P. comunnis		7	Plovdiv, Sofia, Kjustendil
P. armeniaca		2	Petrich
P. persica		11	Sliven
C. pruni	*P. domestica*	287	Sofia, Kjustendil
C. mali	*M. domestica*	1	Sofia, Kjustendil
C. pyri	*P. comunnis*	150	Sofia, Kjustendil, Petrich
C. pyricola	*P. comunnis*	4	Sofia, Kjustendil, Petrich
C. pyrisuga	*P. comunnis*	23	Sofia, Kjustendil, Petrich
C. melanoneura	*M. domestica*	41	Sofia, Kjustendil

DNA extraction and PCR amplification

DNA was extracted from plants and insects with the modified CTAB-based protocol according to Jarausch *et al.* (2011). In total 50 plant samples and more then 300 insects, collected in 2012 were tested with universal ribosomal primer fU5/rU4 for plants and fU5/P7 (Lorenz *et al.*, 1995; Schneider *et al.*, 1995) for insects. '*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.

Molecular characterization of Bulgarian phytoplasma isolates

'*Ca.* Phytoplasma mali'

The biodiversity of '*Ca.* P. mali' isolates was tested by sequence analyses of the imp gene and by sequence analyses of the hflB gene (Seemüller *et al.* 2010; 2011).

The imp gene was amplified using primers f318B_seq/r318B_seq. (Table 2) described by Seemüller *et al.* (2010). PCR amplification was performed in standard 20 µl reaction volume with 1 µM from each primer (Protocol by Jarausch) and the PCR conditions were 1 min at 95 ^{0}C and 40 cycles of 15 sec at 95 ^{0}C, 20 sec at 57 ^{0}C, 45 sec at 69 ^{0}C followed by 4 min at 72 ^{0}C). The PCR products were separated on a 1% agarose gel and visualized through ethidium bromide under UV light. 1 µl from the PCR product was ligated (see below).
The PCR amplification of the hflB gene was performed also in 20 µl reaction volume with 1 µM from primers fHflB3_1/ rHflB4_1 and the PCR amplification parameters were 1 min at 95 ^{0}C and 40 cycles of 15 sec at 95 ^{0}C, 20 sec at 48 ^{0}C, 1 min at 72 ^{0}C followed by 4 min at 72 ^{0}C. Nested amplification with primers fHflB3_1/rHflB3 was done from 1 µl of the first amplification product by the same parameters but by 20 cycles. The PCR products were separated on a 1% agarose gel and visualized through ethidium bromide under UV light. 1 µl from the nested PCR was ligated.

Subtyping of '*Ca*. P. mali' isolates

For the subtyping of '*Ca*. P. mali' in total 23 isolates from different regions in Bulgaria were used (see Table 1). The subtyping of '*Ca*. P. mali' isolates was carried out by PCR-RFLP on the nitroreductase gene according to Jarausch *et al.* (2000). PCR amplification was performed in 30 µl reaction volume with 1 µM from primer pears AP3/AP15 and the PCR amplification parameters were 40 cycles 15 sec at 95 ^{0}C, 20 sec at 60 ^{0}C, 1 min at 69 ^{0}C (initial denaturation and final extension were as above). RFLP analysis on the PCR products was done by digesting 10 µl of the product with BspHI and further 10 µl of the product with Taq I. The restriction fragments were separated on a 2% agarose gel, visualized through ethidium bromide under UV light and the RFLP profiles were compared to reference samples.

'*Ca.* Phytoplasma prunorum'

For molecular characterisation of '*Ca.* P. prunorum' isolates primer pairs (Table 2) for the amplification of aceF and imp genes according to Danet *et al.* (2011) were used. The PCR amplification of aceF and imp gene was performed also in 20 µl reaction volume with 1 µM from each primer and the PCR amplification parameters were 1 min at 95 ^{0}C and 40 cycles of 30 sec at 94 ^{0}C, 30 sec at 50 ^{0}C, 45 sec at 66 ^{0}C followed by a final extension step for 7 min at 66 ^{0}C. 1 µl from the PCR product was ligated.

Table 2 List of primers used in the present study according to Danet *et al.* (2011) and Seemüller *et al.* (2010)

Gene	Length (bp)	Primers	Sequence
aceF ESFY/ PD	797	AceF f1/r1	AceFf1 TAAAATTCGCTGATGTTGGCG AceFr1 CATCTTTCAATTCATTAAAACTAG
aceF ESFY/ PD	797	AceF f2/r2	AceFf2: AGGTATTGAAGAAGGAACTG AceFr2: CAACCGCTTTCATAATAAAAG
pnp PD	549	pnp f2/r2	PNP F2: TACAATTAGATATTAAAGTTAAAGG PNP R2: CATTAATTTTAATACATTTCGC
imp ESFY	673	imp f2/r2	IMP F2: CAGTGTATTAAATTAACAATG IMP R2: CCATCATTACAACTAAAACAT
imp AP	670	f318B_seq/r318B_seq	f318B_seq: AATAATGGAAGCAAATCAAC r318B_seq: GGAGTTCAATTTCATCCT
hflB	528	fHflB3_1/ rHflB4_1	nHflB 3-1 TTCATGATAAGCAACCATAC nHflB 4-1 ATTCAATATGCCTTTGGCAC
hflB	528	Nested fHflB3_1/ rHflB3	TTCTAGCTATTCATCGTGAA CGGCGCGATTAGTAGCTCC

'*Ca.* Phytoplasma pyri'

To characterise the Bulgarian '*Ca.* P. pyri' isolates aceF and pnp genes were used as molecular markers according to the protocol of Danet *et al.* (2011). The primer pairs are shown in Table 2. For aceF a nested PCR approach was needed by using primers aceF f1/r1 for the first amplification in 20 µl reaction volume with 1 µM of each primer and the PCR amplification of 20 cycles (15 sec at 95 ^{0}C, 30 sec at 50 ^{0}C, and 45 sec at 66 ^{0}C). Nested amplification was done from 1 µl of the first amplification product with the primers aceF f2/r2 by the same parameters but by 35 cycles. 1 µl from the nested PCR product was ligated

Cloning, sequencing and phylogenetic analysis

For each molecular marker 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.

Results and discussion:

The results obtained for the detection and identification of phytoplasmas in Bulgarian samples from plants and insects are summarised in Table 3. All three European fruit tree phytoplasmas were found. The results indicate a widespread diffusion of these diseases in Bulgaria.

Table 3. Results of phytoplasma detection by universal- and species-specific primers

Plant/Insect spp.	Host plants	Samples	Phytoplasma type	PCR test positive/negative			
				fu5/ru4	ECA1/ECA2	fPD/rPD	AP3/AP4
M. domestica		29	'*Ca.* P. mali'	24/5	-	-	14/0
P. domestica		27	'*Ca.* P. prunorum'	18/9	18/9	-	-
P. comunnis		17	'*Ca.* P. pyri'	15/2	-	14/3	-
P. armeniaca		2	'*Ca.* P. prunorum'	0/2	-	-	-
P. persica		11	'*Ca.* P. prunorum'	2/9	-	-	-
C. pruni	*P. domestica*	289	'*Ca.* P. prunorum'	10/279	10	-	-
C. mali	*M. domestica*	1	'*Ca.* P. mali'	0/1	-	-	-
C. pyri	*P. comunnis*	150	'*Ca.* P. pyri'	1/149	-	1	-
C. pyricola	*P. comunnis*	4	'*Ca.* P. pyri'	0/4	-	-	-
C. pyrisuga	*P. comunnis*	23	'*Ca.* P. pyri'	0/23	-	-	-
C. melanoneura	*M. domestica*	41	'*Ca.* P. mali'	0/41	-	-	-

Subtyping of '*Ca*. P. mali'.

For a first characterisation of the Bulgarian isolates of '*Ca*. P. mali' the subtyping published by Jarausch *et al.* (2000) was used because it is so far the most used. The results showed that, all tested Bulgarian isolates are from AT-1 type, indigent that they are collected in three completely different and geographically isolated regions. This subtype is the least common in major apple growing regions of Europe (France, Germany, and Italy). Also in samples of Bosnia-Herzegovina the AP subtype was dominant. However, Bulgarian isolates of '*Ca.* P. mali' need to be analyzed to confirm this result.

Multilocus sequence typing (MLST) of Bulgarian isolates of '*Ca*. P. mali', '*Ca*. P. prunorum' and '*Ca*. P. pyri'

In total, 12 isolates of '*Ca*. P. mali', 10 isolates of '*Ca*. P. prunorum' and 7 isolates of '*Ca*. P. pyri' were genetically characterized. A summary is given in Tab. 4. Only the most informative while most polymorphic markers were analyzed. Cloning of PCR products of the different markers was applied in order to identify mixed infections in the trees. Up to three clones per isolate/marker combination were sequenced. Therefore, the number of sequences is higher than the number of samples revealing a high degree of genetic variability of the phytoplasma colonizing a single tree. All obtained sequences for the different markers grouped well with the published sequences of the respective phytoplasma *Candidatus* species.

Table 4. Results of molecular characterization of Bulgarian isolates of '*Ca*. P. mali', '*Ca*. P. prunorum' and '*Ca*. P. pyri'

Number of isolates	Phytoplasma pathogen	Gene marker	Number of sequences in total	Number of unique sequences
10	'*Ca*. P. prunorum'	aceF	19	11
10	'*Ca*. P. prunorum'	imp	20	8
8	'*Ca*. P. mali'	imp	11	9
12	'*Ca*. P. mali'	hflB	24	15
6	'*Ca*. P. pyri'	pnp	6	4
7	'*Ca*. P. pyri'	aceF	7	4

The major part of the obtained sequences has no direct correspondence to published sequences available in GeneBank and many new sequence variants were identified. More time is needed to analyse and confirm the data. A comparison of the types with the data published by Danet *et al.* (2011) was difficult and, thus, a phylogeographic characterisation of the Bulgarian isolates will be more difficult than expected. The first analyses completed in the framework of this STSM indicated, however, a high degree of particularity of Bulgarian fruit tree phytoplasma isolates. The collaboration between both institutes will therefore be continued to further elucidate the phylogeographic situation of Bulgarian isolates and to understand the past and recent spread of fruit tree phytoplasmas in this country.

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