COST Action FA0807

Integrated Management of Phytoplasma Epidemics in Different Crop Systems

Short-term Scientific Mission (STSM) Report

STSM Grantee: Zhelyu Avramov, z.avramov@nsrz.government.bg,
Central Laboratory for Plant Quarantine and Plant Protection Institute – Kostinbrod, Bulgaria

STSM Host: Dr. Xavier Foissac, foissac@bordeaux.inra.fr
UMR1332 Biologie du fruit et Pathologie
INRA and University of Bordeaux (France)

STSM Topic: Genetic diversity of BN phytoplasmas infecting the vineyard and insect vectors in Bulgaria.

COST STSM Reference Number: COST-STSM-FA0807-8246

Grant letter: COST-STSM-ECOST-STSM-FA0807-260911-008066

STSM dates: from 26.09.2011 to 15.10.2011

Purpose of the visit

The main objectives of this scientific mission was to evaluate the genetic diversity of BN phytoplasmas infecting the vineyard and insect vectors in Bulgaria as well as alder yellows phytoplasmas of group 16SrV. This was achieved using molecular detection methods, sequencing and phylogenetic analysis.

Description of the work

DNA extracts from 72 samples collected in 2008, 2009 and 2010 from grapevine (50), insects (10) and alder (12) in Bulgaria were shipped to the INRA laboratories. Preliminary detection was carried out performing PCR assays with universal 16S rDNA primers P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995), followed by primers U5/U3 (Lorenz *et al*., 1995). All of the 70 samples were infected by Bois Noir (70) or Alder Yellows (10) phytoplasmas (tab. 1). Reference isolates for the molecular characterization analyses were kindly provided by the INRA collection. Once the detection of phytoplasmas of the 16SrV and 16SrXII-A groups was confirmed, molecular characterization of the isolates was performed on six different non-ribosomal genes: *map*, *deg*V and *vmp*A for the 16SrV isolates, and *stamp*, *sec*Y and *vmp*1 for 16SrXII-A ones.
Map and *sec*Y are housekeeping genes while DegV is a hypothetical protein gene. *Vmp*A and *vmp*1 are genes encoding membrane proteins and *stamp* is a gene encoding the antigenic membrane protein of stolbur phytoplasma. Since *vmp*A, *vmp*1 and *stamp* genes encode membrane proteins, they are supposed to present a higher variability than housekeeping genes. Amplification of the six non-ribosomal genes was carried out by nested PCR with the primers described in Tab. 2.
PCR mixtures contained dNTP - 5mM of each, 25 mM of $MgCl_2$, 100 mM of each primer, Taq buffer (10x) and 0.04µl /µl mix of Taq enzyme (Taq Polymerase: M0273X BioLabs). 1 µl of DNA was used in each reaction. Final volume was 25 µl for the first PCR and 50 µl for the nested reaction.

RFLP analysis was performed for *map* and *vmp*A amplicons. 20 ul the enzymatic digestion mixture contained 1 ul of each enzyme (10 U/ul), 2 ul of 10x buffer and 10 ul of PCR product. Digested PCR products were then loaded on 3% agarose gel, stained with ethidium bromide and visualized on a transilluminator after electrophoresis.

Code		Year	Hosts	Region	16S rDNA	Code		Year	Hosts	Region	16S rDNA
BG	5619	2009	grapevine	V. Turnovo	+	BG	305	2009	grapevine	Shumen	+
BG	5839	2009	grapevine	Lovech	+	BG	5813	2009	grapevine	Shumen	+
BG	5644	2009	grapevine	Targoviste	+	BG	2517	2010	grapevine	Russe	+
BG	5646	2009	grapevine	Targoviste	+	BG	2504	2010	grapevine	Russe	+
BG	5275	2009	grapevine	Russe	+	BG	2350	2010	grapevine	Shumen	+
BG	5386	2009	grapevine	Targoviste	+	BG	2506	2010	grapevine	Russe	+
BG	5250	2009	grapevine	Russe	+	BG	2590	2010	grapevine	Varna	+
BG	5414	2009	grapevine	Bourgas	+	BG	2560	2010	grapevine	Plovdiv	+
BG	5410	2009	grapevine	Bourgas	+	BG	2466	2010	grapevine	Montana	+
BG	1285	2009	grapevine	Russe	+	BG	2507	2010	grapevine	Russe	+
BG	1282	2009	grapevine	Russe	+	BG	329	2011	grapevine	Montana	+
BG	5271	2009	grapevine	Russe	+	BG	1647	2011	grapevine	Plovdiv	+
BG	5051	2009	grapevine	Sliven	+	BG	4560	2009	grapevine	Shumen	+
BG	5054	2009	grapevine	Sliven	+	BG	2863	2009	*Hyalestes obsoletus*	Pleven	+
BG	4911	2009	grapevine	Plovdiv	+	BG	3032	2009	*Hyalestes obsoletus*	Sliven	+
BG	4921	2009	grapevine	Plovdiv	+	BG	3154	2009	*Hyalestes obsoletus*	Varna	+
BG	4794	2009	grapevine	Plovdiv	+	BG	1455	2010	*Reptalus panzeri*	Pleven	+
BG	5113	2009	grapevine	Plovdiv	+	BG	1459	2010	*Reptalus panzeri*	Pleven	+
BG	5687	2009	grapevine	Sliven	+	BG	1452	2010	*Hyalestes obsoletus*	Komostica	+
BG	4263	2009	grapevine	Bourgas	+	BG	1453	2010	*Reptalus panzeri*	Bezdenitsa	+
BG	5683	2009	grapevine	Bourgas	+	BG	1435	2010	*Reptalus spp.*	V. Turnovo	+
BG	5794	2009	grapevine	Sliven	+	BG	1436	2010	*Reptalus panzeri*	V. Turnovo	+
BG	4258	2009	grapevine	Bourgas	+	BG	1816	2010	*Reptalus panzeri*	Nedialsko	+
BG	5204	2009	grapevine	V. Turnovo	+	BG	1840	2010	*Reptalus panzeri*	Nikolaevo	+
BG	5428	2009	grapevine	Chumen	+	BG	1212	2011	*Scaphoideus titanus*	Vratsa	-
BG	4245	2009	grapevine	Bourgas	+	BG	1179	2010	Alnus glutinosa	Samokov	+
BG	5663	2009	grapevine	Targoviste	+	BG	1180	2010	Alnus glutinosa	Samokov	+
BG	5205	2009	grapevine	V. Turnovo	+	BG	1181	2010	Alnus glutinosa	Samokov	+
BG	5681	2009	grapevine	Bourgas	+	BG	831	2011	Alnus incana	Blagoevgrad	+
BG	4247	2009	grapevine	Bourgas	+	BG	832	2011	Alnus incana	Blagoevgrad	+
BG	4747	2009	grapevine	Razgrad	+	BG	833	2011	Alnus glutinosa	Blagoevgrad	+
BG	4143	2009	grapevine	Bourgas	+	BG	835	2011	Alnus glutinosa	Blagoevgrad	-
BG	4144	2009	grapevine	Bourgas	+	BG	1211	2011	Alnus incana	Kjustendil	+
BG	342	2009	grapevine	Targoviste	+	BG	1312	2011	Alnus incana	Kjustendil	+
BG	3041	2009	grapevine	V. Turnovo	+	BG	1413	2011	Alnus glutinosa	Kjustendil	+
BG	5205d	2009	grapevine	Bourgas	+	BG	1474	2011	Alnus glutinosa	Russe	+

Tab.1. List of the samples from Bulgaria with the preliminary results.

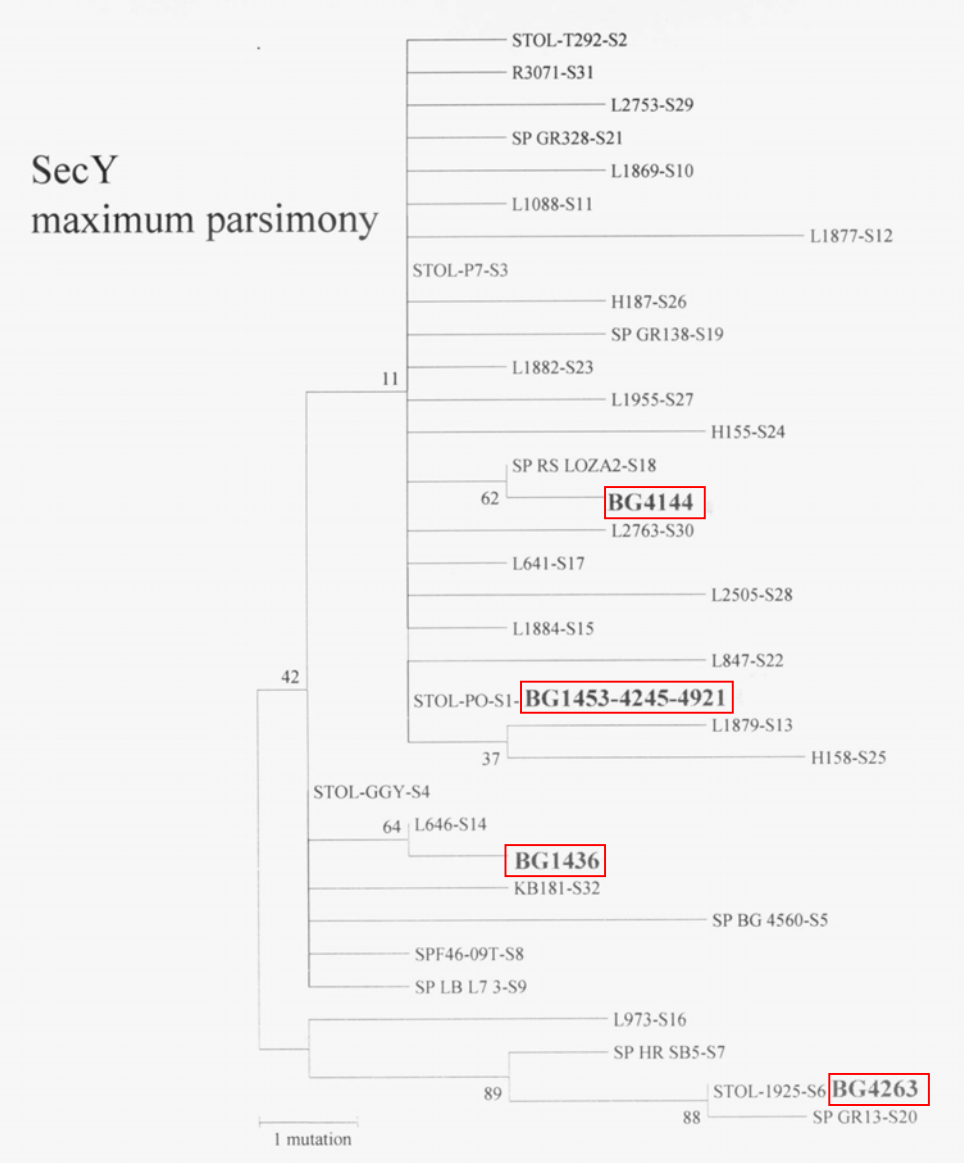
Amplicons obtained after nested PCR for *map*, *vmp*A, *sec*Y and *stamp* from a variable number of selected samples were sequenced and a phylogenetic tree including reference sequences and BN from Tuscany was generated for each gene. PCR products were analyzed with Capillary electrophoresis (Qiaxcel apparatus). All positive PCR products were submitted to sequences in BECKMAN-COULTER GENOMICS Company in United Kingdom. The sequences obtained were assembled and edited with Pregap4 and Gap4 softwares. Alignment of the sequences and creation of a phylogenetic tree (maximum parsimony method) were performed with Mega5 software.

Primers sets
***Map* gene**
1st PCR FD9f5 / Mapr1 (Arnaud et al., 2007)
Nested PCR FD9f6 / Mapr2 (Arnaud et al., 2007)
***Deg*V gene**
1st PCR UVRBf1 / DEGVr4 (Arnaud et al., 2007)
Nested PCR UVRBf3 / DEGVr3 (Arnaud et al., 2007)
***Vmp*A gene**
1st PCR FD92f5 / FD92r3 (Foissac et al, unpublished)
Nested PCR FD92f8 / FD92r7 (Foissac et al, unpublished)
Sequencing FD92f3 and FD92r5 (Foissac et al, unpublished)
***Vmp*1 gene**
1st PCR STOLH10F1 / STOLH10R1 (Foissac et al, unpublished)
Nested PCR TYPH10F / TYPH10R (Foissac et al, unpublished)
***Sec*Y gene**
1st PCR PosecF1 / PosecR1 (Fialova et al., 2009)
Nested PCR Posec N2 / Posec R3 (Foissac et al., unpublished)
***Stamp* gene**
1st PCR StampF / StampR0 (Fabre et al., 2011)
Nested PCR StampF1/ StampR1 (Fabre et al., 2011)

Tab.2 Genes and Primer sets Primer sets used for the amplification and sequencing of Map, DegV, VmpA, Vmp1, SecY and Stamp genes.

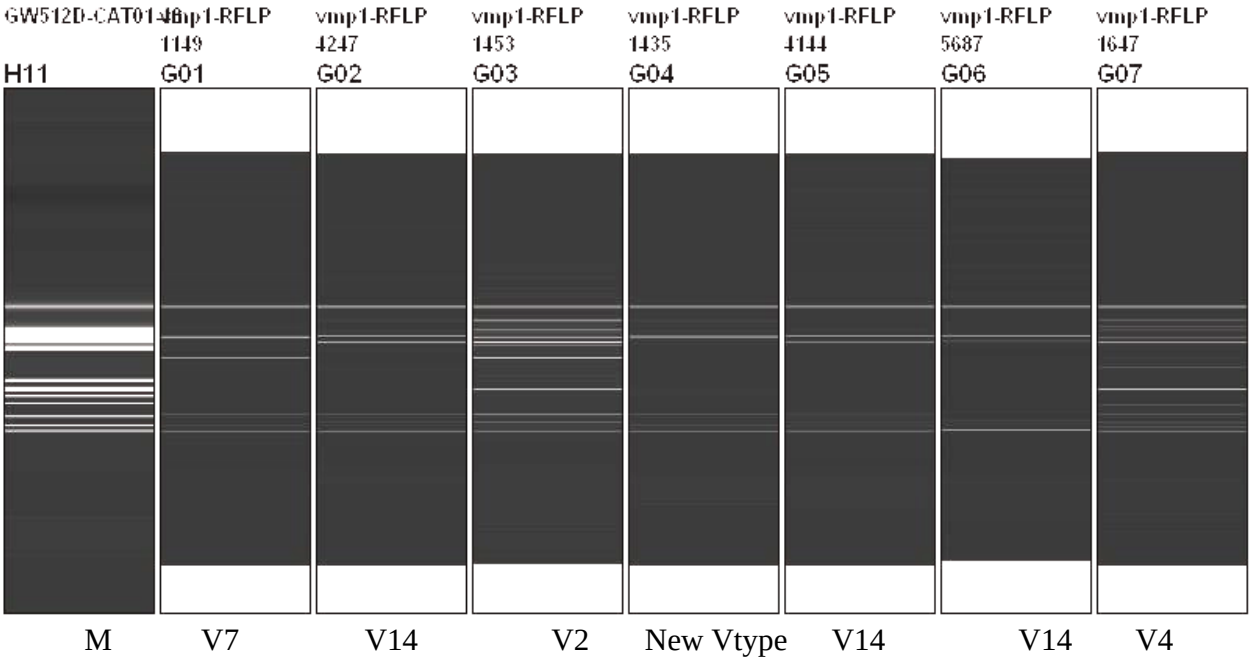
Description of the main results obtained

*Sec*Y: Seven DNA samples from grapevines were shown positive results by secY gene testing. They were selected for registering and preparing to be sequenced. Sequence analysis showed that the isolates BG4144, BG1453, BG4245, BG4921, BG1436, BG4560 belong respectively to the genotypes S1, S4, S5, S14 (Tuf type-b) and S18 which are common genotypes propagated from bindweed and BG4263 belong to S6 (Tuf type-a) propagated in western Europe from stinging nettle.

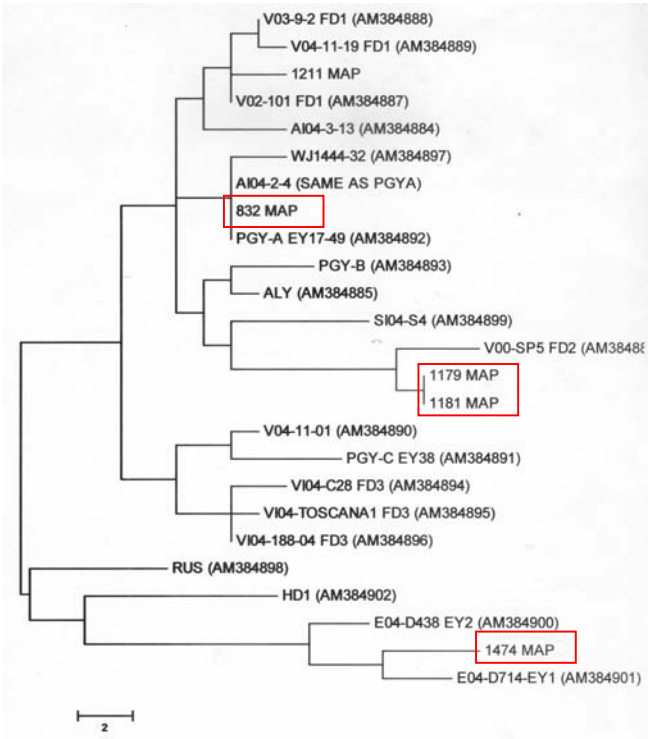


Stamp: After performing of the Stamp gene test 31 samples show positive results. The all samples were subjected for sequencing. Stamp sequences revealed that 5 isolate are of genotype ST11 (tufB cluster II), 13 isolates ST13 (tufB cluster III), 11 isolates is ST9 (tufB cluster II) and 2 isolates are completely different and will be belong to the two new ST genotypes. These genotypes grouped in tufB clusters II, III and IV which corresponds to isolates of South East Europe and East of the Mediterranean basin. So the genetic diversity of stolbur phytoplasma isolates seems to be relatively high in Bulgaria.

*Vmp*1: Twelve isolates shown positive results after Vmp1 testing. The following isolates BG4911, BG4247, BG4144, BG5687 BG1453, BG1435 and BG1647 ware prepared and sequenced. The others showed very weak bands of the expected size and were not sequenced. This seven samples were characterized by RFLP with the enzyme *Rsa*I and they belong to the genotype V7 (1isolates), V14 (tree isolates), V2 (one isolate), V4 (one isolate). These genotypes are quite common in Europe. The isolate BG1435 shown completely new profile and it will be tested again.



The samples from alder were submitted to *Map, Deg*V. First PCR FD9F5 / MAPR1; Second PCR FD9F6/MAPR2. Volume of 0.5µl of DNA for first PCR and 0.5µl PCR product for nested PCR were used; PCR reactions were performing in a final total volume of 25 µl for the first PCR and 50 µl for the nested reaction. The PCR products were analyzed with Capillary electrophoresis (Qiaxcel apparatus). The all twelve samples show positive results and all of them was sequencing. Two out of four samples from alder belong to *map*-FD2 group – BG 1179, BG1181, one to FD1 – BG1211 and one sample belong to EY1. Maybe the sample is from elm not from alder.



*Vmp*A gene for Alder. *Vmp*A gene was amplified from all of the 12 alder samples. Considering *Vmp*A is variable in size, PCR products from 4 samples showing were chosen for sequencing with primer pair FD92F3/R5. The analysis of the sequences was not achieved during the period of the mission and will be analysed in the following next weeks.

Future collaboration with host institution

The results obtained from this scientific mission allowed the start of the molecular characterization of the phytoplasmas infecting vineyards in Bulgaria. I am planning to apply the techniques I learned during this period to a wider range of phytoplasma isolates from Bulgaria and I hope to be participating in future in more exhaustive projects about phytoplasma molecular characterization in collaboration with the host institution.