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

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UMR1332 Biologie du fruit et Pathologie
INRA and University of Bordeaux (France)
STSM Topic: Multilocus sequence typing of *Bois noir* (BN) isolates collected in Croatia

Reference codes: STSM: COST-STSM-FA0807-10423
Grant letter: COST-STSM-ECOST-STSM-FA0807-200512-016892
STSM dates: 20 May 2012 to 02 June 2012

Purpose of the visit

The topic of this visit was molecular characterization of *Bois noir* (BN) phytoplasmas infecting vineyards in Croatia as well as molecular characterization of some BN samples collected in the frame of the SEE-ERA.NET project. Non-ribosomal genes (*tuf, vmp1, secY, stamp*) together with 16S rRNA gene are currently proposed as genetic markers for finer differentiation of stolbur phytoplasmas. Throughout typing, sequencing and phylogenetic analysis of these non-ribosomal genes the aim was to investigate genetic diversity of stolbur phytoplasma strains in Croatia and West Balkan Countrys.

Description of the work

DNA extracts from samples collected in Croatian winegrowing regions collected during 2009. and 2010. together with samples collected in the frame of SEE-ERA.NET project from different plant hosts and insect vectors in which the presence of *Bois noir* (BN) phytoplasmas was previously demonstrated, were used for multigene characterization on 5 different non-ribosomal genes: *tuf* (Langer and Maixner, 2004; Foissac and Fabre unpublished), *secY, vmp*1 (Cimerman *et al*., 2009; Fialova *et al.,* 2009), *vmp*3 (Foissac, unpublished) and *stamp* (Fabre *et al*., 2011). From samples collected in the frame of SEE-ERA.NET project only those that needed additional typing are chosen for analysis. Reference strains (maintained in periwinkle) employed, were from the Collection of INRA-Bordeaux, France.

Amplification of the genes was carried out by nested PCR with the primers described in Table 1.

Table 1. Primer sets used for molecular characterization of stolbur samples

Gene		Primer sets
Stamp	1st PCR Nested PCR	StampF / StampR0 (Fabre *et al*., 2011) StampF1/ StampR1 (Fabre *et al*., 2011)
Vmp1	1st PCR Nested PCR	STOLH10F1 / STOLH10R1 (Cimerman *et al.*, 2009) TYPH10F / TYPH10R (Fialova *et al*., 2009)
SecY	1st PCR Nested PCR	PosecF1 / PosecR1 (Fialova *et al*., 2009) Posec N2 / Posec R3 (Danet *et al*., unpublished)
Tuf	1st PCR Nested PCR	ftuf1 / rtuf1 ftufAY / rtufStol (Langer and Maixner, 2004; Foissac and Fabre unpublished)
Vmp3	1st PCR Nested PCR	Vmp3-F5 /Vmp3-R4 Vmp3-F6 /Vmp3-R6 (Salar and Foissac, unpublished)

Results

tuf

More than 60 selected stolbur isolates from grapevine, crops, weeds and insects where subjected to *tuf* gene analysis by direct and nested PCR with the primers described in Table 1.
Amplicons obtained after nested PCR were sent for sequencing. Sequence analysis was not finished during the period of the scientific mission and will be analysed in the following weeks.

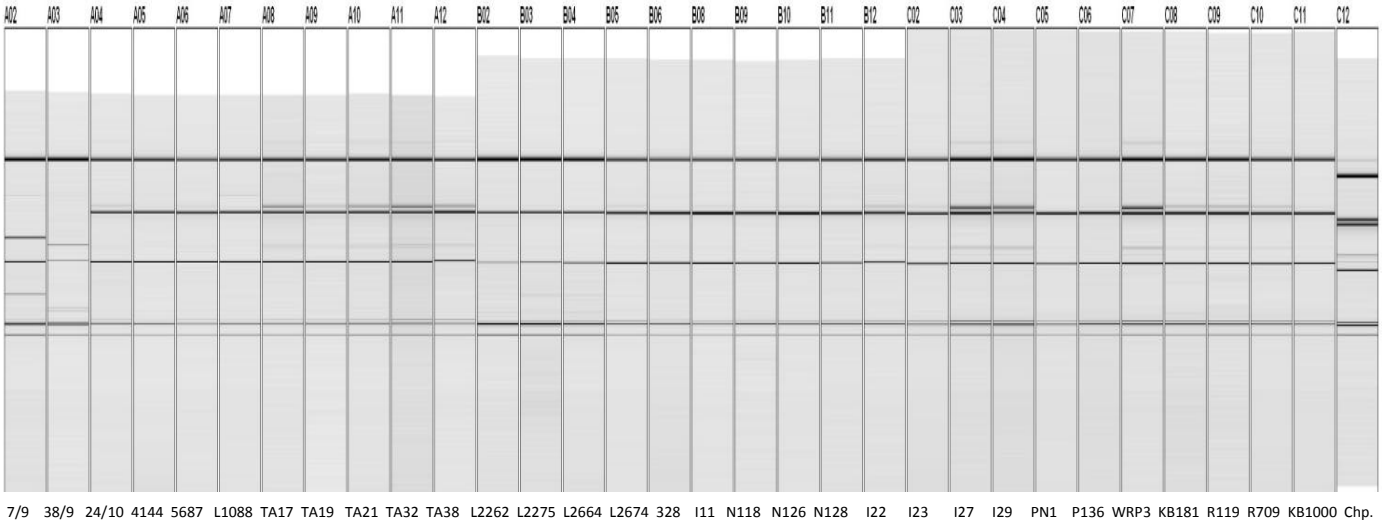
Table 2. Results of nested PCR assay for *tuf* gene

F	G	3141	3143	3169	3148	SRS16	27188	30003	33060
35869	35922	846	1174	1175	Pot4	Pot2	Pot5	Pot4b	Egy17
06-21-5	08-25-13	L2667	L3981	L3991	L4009	L4222	L4300	2393	3331
3340	3878	4433	Char.2	364	P179	I31	PN5	2-9	18-9
7-10	37-10	40-10	Ch1	IP5	IP6	PI34	PI38	ORT2	YON3
300	34	Loza149	Loza196	P324	PF246	PF260	19-9	Ho4MNS	KB146B
KB194/2	R2063	R597	R9000	Rp1MSS	Rp2MSS	33429	34246	IP12	Ho10MNS
ZG-2	VU-8	VZ-1	DEP	E	11-10	7-9	Nk	Ps	PO

red: positive samples sent for sequencing
red: positive samples sent for sequencing (Croatian samples)
black: negative samples

In addition to nested PCR assay further characterization was carried out on *tuf* gene for another part of the samples (tab.3). RFLP analysis with *Hpa*II enzyme of nested PCR products ftufAY/rtufStol (Langer and Maixner, 2004) showed that all the samples except two Croatian samples (7/9 and 38/9) belong to tuf-type II. Sample 7/9 has tuf-type I profile while the profile of sample 38/9 could not be determined.

Figure 1. RFLP *tuf/HpaII* results on BN infected samples from Croatia and SEE-ERA.NET project



All PCR products were analyzed with capillary electrophoresis (Qiaxcel).

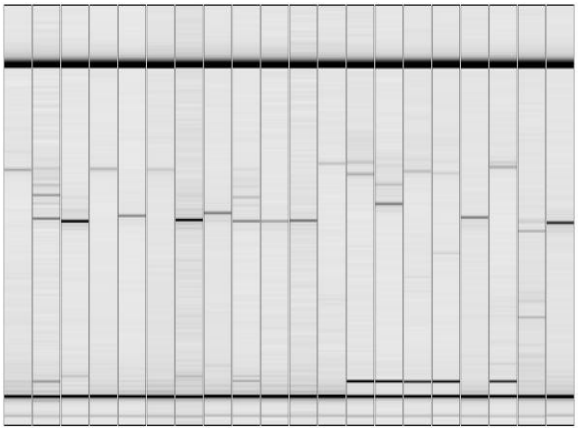
Table 3. List of samples from Croatia and See-eranet project analysed by RFLP *tuf/HpaII*

Species	Country code	Marker tufAY	Lab code
Vitis vinifera	HR	T2	24-10
Vitis vinifera	HR	T1	7-9
Vitis vinifera	HR	Not determined	38-9
Vitis vinifera	BG	T2	4144
Vitis vinifera	BG	T2	5687
Lamiaceae Lavandula	F	T2	L1088
Nicotiana tabacum	F	T2	TA17
Nicotiana tabacum	F	T2	TA19
Nicotiana tabacum	F	T2	TA21
Nicotiana tabacum	F	T2	TA32
Nicotiana tabacum	F	T2	TA38
Lamiaceae Lavandula	F	T2	L2262
Lamiaceae Lavandula	F	T2	L2275
Lamiaceae Lavandula	F	T2	L2664
Lamiaceae Lavandula	F	T2	L2674
Capsicum annum	GR	T2	328
Vitis vinifera	H	T2	I11
Solanum tuberosum	H	T2	N118
Solanum tuberosum	H	T2	N126
Solanum tuberosum	H	T2	N128
Capsicum annum	H	T2	I22
Capsicum annum	H	T2	I23
Solanum lycopersicum	H	T2	I27
Solanum lycopersicum	H	T2	I29
Vitis vinifera	H	T2	PN1
Solanum tuberosum	RO	T2	P136
Reptalus panzeri inoculated periwinkle	RS	T2	WRP3
Reptalus panzeri	RO	T2	KB181/3
Reptalus quinquecostatus	RO	T2	R119
Reptalus quinquecostatus	RO	T2	R709
Hyalesthes obsoletus	RU	T2	KB1000
Catharanthus roseus	F	T2	Champlong

vmp3

One additional hyper variable marker – *vmp3* was chosen for study of genetic diversity of stolbur phytoplasmas. *Vmp3* is a variable membrane protein gene with a C-terminal part containing collagen-like (GXY) repeats, number of which varies from one strain to another. However, since new primers in this combination (tab.1) were employed for the first time results were not satisfactory and require further optimization of the protocol (fig.2).

Figure 2. Results of *vmp3* nested-PCR

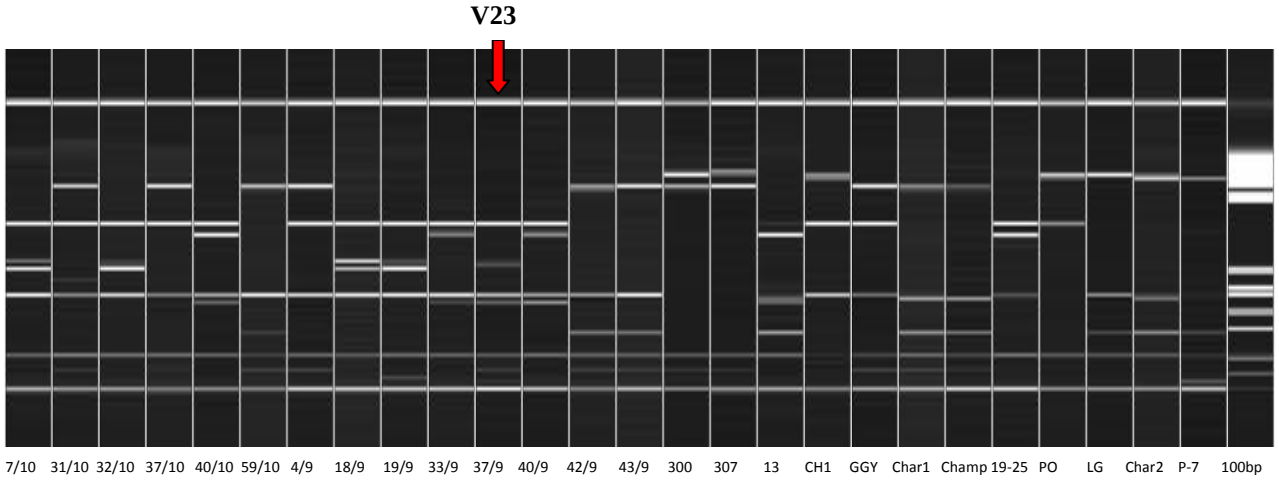


vmp1

Selected samples (21) collected in croatian vine regions were used to study variability in the *vmp1* gene. *Vmp1* gene encodes a putative membrane protein and therefore shows higher variability than housekeeping genes and represents a suitable marker for molecular epidemiology. *Vmp1* gene was amplified by direct and nested PCR with the primers described in Table 1. Positive *vmp1* PCR products were submitted to *RsaI* digestion at 37°C.

Different profiles, including one new profile (V23) were identified in the examined samples. Samples were chosen for sequencing according to their profile with primer pair TYPH10F/ TYPH10R (fig.3).

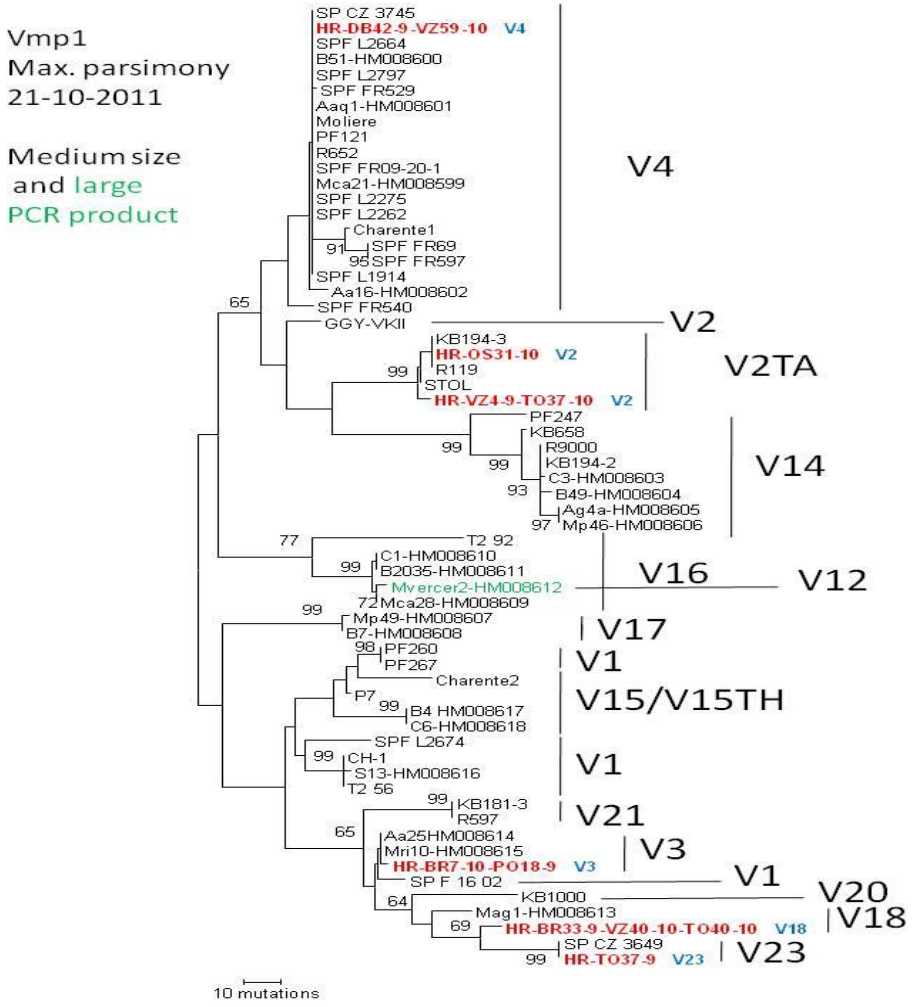
Figure 3. RFLP *vmp1/RsaI* results on BN infected samples from Croatia



Among 15 Croatian BN isolates, 5 different RFLP types obtained by *RsaI* digestion of *vmp1* gene sequences were found - V2, V3, V4, V18 and new genotype V23.

A phylogenetic tree was generated including selected Croatian samples that were sequenced together with reference sequences (fig.4).
The sequences were assembled and edited with the Staden package (Pregap4 and Gap4) or the Phred-Phrap-Consed package. Alignment of the sequences and reconstruction of phylogenetic trees (maximum parsimony or UPGMA methods) were performed with the Mega5 software.

Figure 4. Phylogenetic tree of the vmp1 gene sequences



secY* and *STAMP

The presence of phytoplasma infection was also investigated on 2 other non-ribosomal genes: House-keeping gene *sec*Y and gene encoding the antigenic membrane protein *STAMP*. Amplification of these two genes was carried out by nested PCR with the primers previously described (tab.1).
Only few selected samples were used for this molecular characterization.

Conclusion

In conclusion, PCR and RFLP analysis supported by nucleotide sequencing allows us to distinguished different stolbur genotypes and start to survey their geographical distribution in Croatia. The perpective of this work is to determine the plant reservoir and insect vectors of the different stolbur-BN genotypes present in Croatia in order to improve BN disease control.