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

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Purpose of the visit
The topic of my stage at INRA was the molecular characterization of phytoplasmas infecting vineyards in Tuscany. I was aiming to acquire competence on the techniques used for molecular characterization, such as Nested PCR and RFLP analysis, and to learn using bioinformatics tools for sequencing and phylogenetic analysis.

Description of the work
DNA extracts from 68 samples collected in 2010 from grapevine (59) and alder (9) in Tuscany were shipped to the INRA laboratories. All of the 68 samples previously proved to be infected by Flavescence doree (21), Bois Noir (38) or Alder Yellows (9) phytoplasmas. In particular, all of the 21 FD isolates belonged to the 16SrV-C subgroup, while BN isolates belonged to both Tuf-a and Tuf-b types (tab 1).
Reference isolates for the molecular characterization analyses were kindly provided by the UMR 1332 "Biologie du Fruit et Pathologie".
First of all, the presence of phytoplasma infection was checked with a Triplex Real Time assay for the detection of 16SrV and 16SrXII-A groups phytoplasmas and an endogenous control (Pelletier *et al.*, 2009). All of the 68 samples resulted positive to 16Sr-V (30) or 16Sr-XII-a (38) phytoplasma groups. Two samples (AL6 and PI50bis), showing high Ct values, were discarded for the following analyses.
Once the detection of 16SrV and 16SrXII-A groups phytoplasmas was confirmed, molecular characterization of the isolates was performed on six different non-ribosomal genes: Map, DegV and vmpA for the 16SrV isolates, and Stamp, SecY and vmp1 for 16SrXII-A ones.
Map and SecY are housekeeping genes while DegV is a hypothetical protein gene. VmpA and vmp1 are genes encoding membrane proteins and Stamp is a gene encoding the antigenic membrane protein of stolbur phytoplasma. Since VmpA, Vmp1 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 5uM of each dNTP, 25 mM of MgCl2, 100 uM of each primer, Taq buffer (10x) and 0,008 U/ul of Taq enzyme (aTaq DNA Polymerase, Promega). 1 ul of diluted (1:10) DNA was used in each reaction.
Final volume was 25 ul for the first PCR and 50 ul for the nested reaction.
RFLP analysis was performed for Map and VmpA 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	(host)	origin	16SrV	16SrXII-A
LU 36	*(V. vinifera)*	Lucca	+ (V-C)	-
LU 38	*(V. vinifera)*	Lucca	+ (V-C)	-
LU 39	*(V. vinifera)*	Lucca	+ (V-C)	-
LU 40	*(V. vinifera)*	Lucca	+ (V-C)	-
LU 40 bis 2	*(V. vinifera)*	Lucca	+ (V-C)	-
LU 52	*(V. vinifera)*	Lucca	+ (V-C)	-
LU 53	*(V. vinifera)*	Lucca	+ (V-C)	-
LU 54	*(V. vinifera)*	Lucca	+ (V-C)	-
LU 56	*(V. vinifera)*	Lucca	+ (V-C)	-
LU 57	*(V. vinifera)*	Lucca	+ (V-C)	-
LU 66	*(V. vinifera)*	Lucca	+ (V-C)	-
LU 67	*(V. vinifera)*	Lucca	+ (V-C)	-
LU 68	*(V. vinifera)*	Lucca	+ (V-C)	-
LU 70	*(V. vinifera)*	Lucca	+ (V-C)	-
LU 70 bis	*(V. vinifera)*	Lucca	+ (V-C)	-
LU 70 bis 2	*(V. vinifera)*	Lucca	+ (V-C)	-
LU 95 bis	*(V. vinifera)*	Lucca	+ (V-C)	-
MS 57	*(V. vinifera)*	Massa-Carrara	+ (V-C)	-
MS 58	*(V. vinifera)*	Massa-Carrara	+ (V-C)	-
MS 59	*(V. vinifera)*	Massa-Carrara	+ (V-C)	-
MS 94	*(V. vinifera)*	Massa-Carrara	+ (V-C)	-
AL 1	*(A. glutinosa)*	Pisa	+	-
AL 2	*(A. glutinosa)*	Pisa	+	-
AL 3	*(A. glutinosa)*	Pisa	+	-
AL 4	*(A. glutinosa)*	Pisa	+	-
AL 5	*(A. glutinosa)*	Pisa	+	-
AL 6	*(A. glutinosa)*	Pisa	+	-
AL 7	*(A. glutinosa)*	Pisa	+	-
AL 8	*(A. glutinosa)*	Pisa	+	-
AL 9	*(A. glutinosa)*	Pisa	+	-
MS 62	*(V. vinifera)*	Massa-Carrara	-	+ (Tuf-a)
MS 65 bis	*(V. vinifera)*	Massa-Carrara	-	+ (Tuf-b)
MS 95	*(V. vinifera)*	Massa-Carrara	-	+ (Tuf-a)
Ms 85bis	*(V. vinifera)*	Massa-Carrara	-	+ (Tuf-a)

Code	(host)	origin	16Sr V	16SrXII-A
MS 72	*(V. vinifera)*	Massa-Carrara	-	+ (Tuf-a)
MS 71	*(V. vinifera)*	Massa-Carrara	-	+ (Tuf-a)
PI 35	*(V. vinifera)*	Pisa	-	+ (Tuf-a)
PI 34	*(V. vinifera)*	Pisa	-	+ (Tuf-a)
PI 33	*(V. vinifera)*	Pisa	-	+ (Tuf-a)
PI 31	*(V. vinifera)*	Pisa	-	+ (Tuf-b)
PI 43	*(V. vinifera)*	Pisa	-	+ (Tuf-b)
PI 39	*(V. vinifera)*	Pisa	-	+ (Tuf-b)
PI 44	*(V. vinifera)*	Pisa	-	+ (Tuf-b)
PI 41	*(V. vinifera)*	Pisa	-	+ (Tuf-b)
PI 38	*(V. vinifera)*	Pisa	-	+ (Tuf-b)
PI 50	*(V. vinifera)*	Pisa	-	+ (Tuf-b)
PI 46	*(V. vinifera)*	Pisa	-	+ (Tuf-b)
PI 47	*(V. vinifera)*	Pisa	-	+ (Tuf-a)
PI 50bis	*(V. vinifera)*	Pisa	-	+ (Tuf-b)
PI 48	*(V. vinifera)*	Pisa	-	+ (Tuf-a)
LI 36	*(V. vinifera)*	Livorno	-	+ (Tuf-a)
LI 50	*(V. vinifera)*	Livorno	-	+ (Tuf-b)
LI 44	*(V. vinifera)*	Livorno	-	+ (Tuf-a)
LI 43	*(V. vinifera)*	Livorno	-	+ (Tuf-a)
GR 26	*(V. vinifera)*	Grosseto	-	+ (Tuf-b)
GR 38	*(V. vinifera)*	Grosseto	-	+ (Tuf-b)
GR 24	*(V. vinifera)*	Grosseto	-	+ (Tuf-b)
GR 23	*(V. vinifera)*	Grosseto	-	+ (Tuf-a)
GR 36	*(V. vinifera)*	Grosseto	-	+ (Tuf-b)
GR 37	*(V. vinifera)*	Grosseto	-	+ (Tuf-b)
GR 25	*(V. vinifera)*	Grosseto	-	+ (Tuf-b)
GR 40	*(V. vinifera)*	Grosseto	-	+ (Tuf-b)
GR 29	*(V. vinifera)*	Grosseto	-	+ (Tuf-b)
GR 39	*(V. vinifera)*	Grosseto	-	+ (Tuf-b)
LU 69	*(V. vinifera)*	Lucca	-	+ (Tuf-a)
LU 73	*(V. vinifera)*	Lucca	-	+ (Tuf-a)
LU 84	*(V. vinifera)*	Lucca	-	+ (Tuf-a)
MS 60	*(V. vinifera)*	Massa-Carrara	-	+ (Tuf-b)

Tab.1 List of the samples shipped to the INRA laboratories, including plant host, location of origin and the results of previous diagnosis and characterizationa analyisis.

Amplicons obtained after nested PCR for Map, VmpA, SecY and Stamp from a variable number of selected samples were sequenced and a phylogenetic tree including tuscan and reference sequences was generated for each gene.

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 Mega4 software.

Gene		Primer sets
Map	1st PCR	FD9f5 / Mapr1 (Arnaud et al., 2007)
	Nested PCR	FD9f6 / Mapr2 (Arnaud et al., 2007)
DegV	1st PCR	UVRBf1 / DEGVr4 (Arnaud et al., 2007)
	Nested PCR	UVRBf3 / DEGVr3 (Arnaud et al., 2007)
VmpA	1st PCR	FD92f5 / FD92r3 (Foissac et al, unpublished)
	Nested PCR	FD92f8 / FD92r7 (Foissac et al, unpublished)
	Sequencing	FD92f3 and FD92r5 (Foissac et al, unpublished)
Vmp1	1st PCR	STOLH10F1 / STOLH10R1 (Foissac et al, unpublished)
	Nested PCR	TYPH10F / TYPH10R (Foissac et al, unpublished)
SecY	1st PCR	PosecF1 / PosecR1 (Fialova et al., 2009)
	Nested PCR	Posec N2 / Posec R3 (Foissac et al., unpublished)
Stamp	1st PCR	StampF / StampR0 (Fabre et al., 2011)
	Nested PCR	StampF1/ StampR1 (Fabre et al., 2011)

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

Description of the main results obtained:

16SrV isolates

Map: PCR products were loaded on an agarose gel and visualized with a transilluminator after electrophoresis. Map gene was successfully amplified from 25 out of 29 samples.
PCR products from 7 samples underwent RFLP analyses with enzymes Eco72I and AluI. 6 of 7 samples showed the same profile of reference control FD70, while one (LU57) had the same profile of FDC28 (fig. 1).
The 18 remaining PCR products were sequenced using primer MapR2. Analysis of the sequences showed the tuscan isolates clustering in all of the three main groups: FD1 (reference strain FD70), FD2 (reference strain FD92) and FD3 (reference strain FDC28). The same three groups can be identified by RFLP, with FD70, FD92 and FDC28 showing different profiles.
All the tuscan isolates grouped in group FD2 and FD3 resulted to have exactly the same Map nucleotide sequence, as well as 7 FD1 isolates. 3 FD1 isolates (AL8, AL8 and AL9, all from alders) shared a different Map sequence.

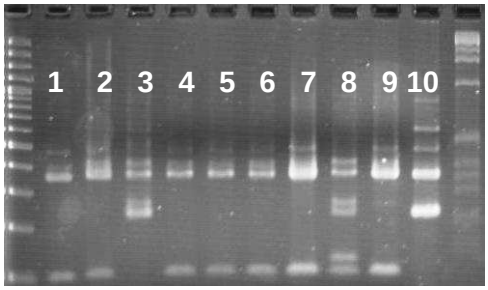

Fig.1 Restriction fragment length polymorphism profiles of Map gene sequences amplified from 7 samples and 3 reference controls. From 1 to 10: LU53, LU54, LU57, LU67, LU70, LU70bis2, MS58, FD92, FD70, C28

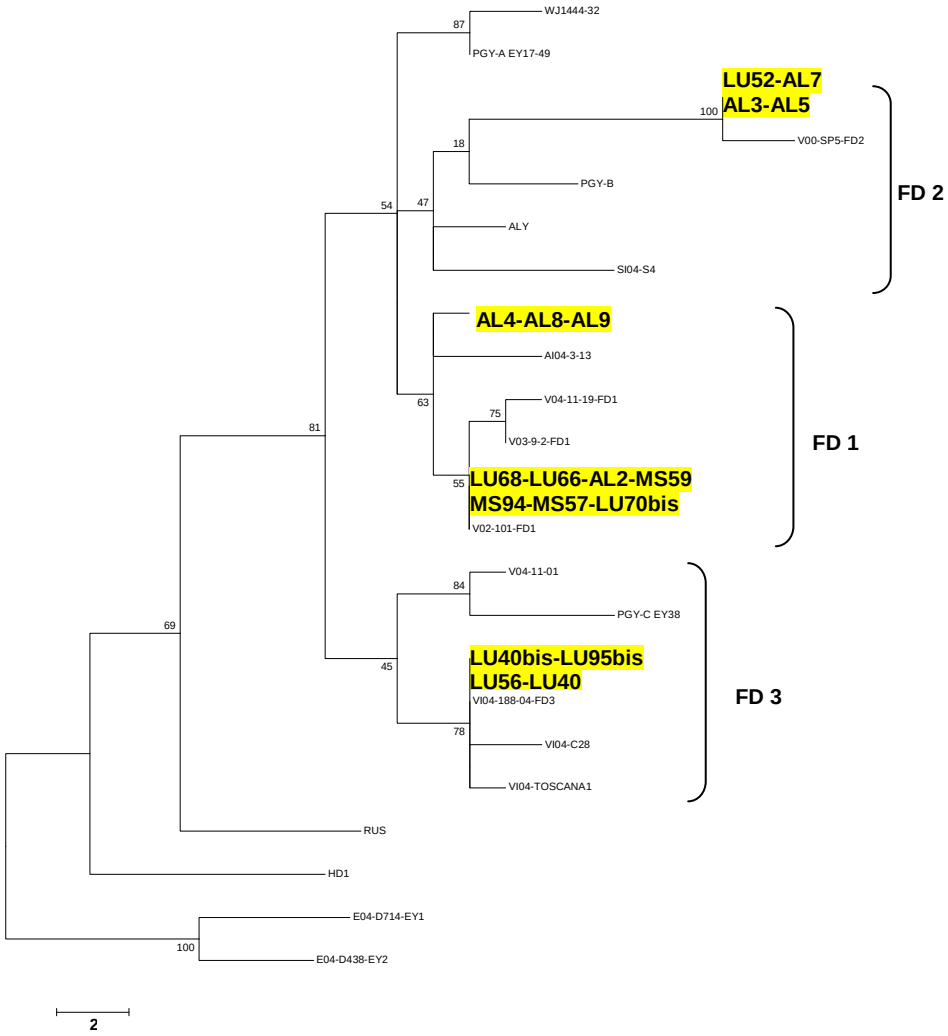

Fig.2 Phylogenetic tree generated using maximum parsimony method with Map sequences.

DegV: The amplification of this gene was problematic. Despite all of the 3 positive controls were successfully amplified, after electrophoresis on agarose gel only 9 out of 29 samples showed bands of the expected size (fig.3)

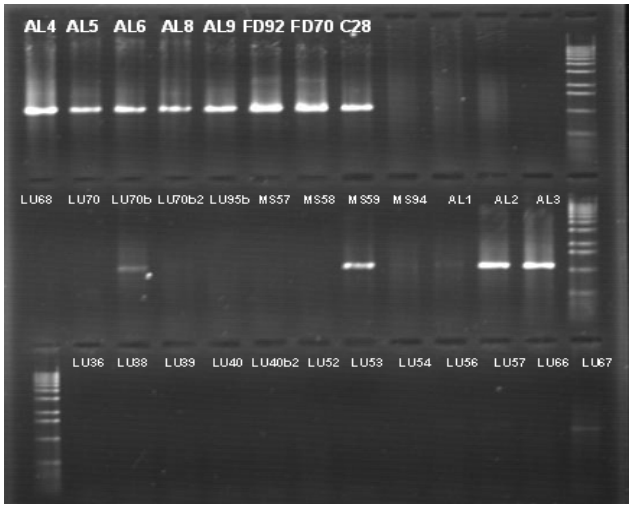

Fig. 3 Amplicons obtained with nested PCR on DegV gene from 29 samples and 3 positive controls

VmpA: VmpA gene was amplified from all of the 29 samples. Considering VmpA is variable in size, PCR products from 18 samples showing different amplicon size were chosen for enzymatic digestion with BfmI and 6 of them were then sequenced with primer pair FD92F3/R5.
According to the RFLP analysis, all of the 18 samples showed the same pattern of reference strains FDC28 and FDCAM (fig. 4 a,b).
The analysis of the sequences showed 5 samples belonging to cluster III (along with FD70, FDC28 and FDCAM reference strains) and 1 (LU 52) belonging to cluster II (along with FD92), but quite different from every other isolate of that cluster (fig. 5). None of the 6 tuscan isolates shared the same VmpA nucleotide sequence.

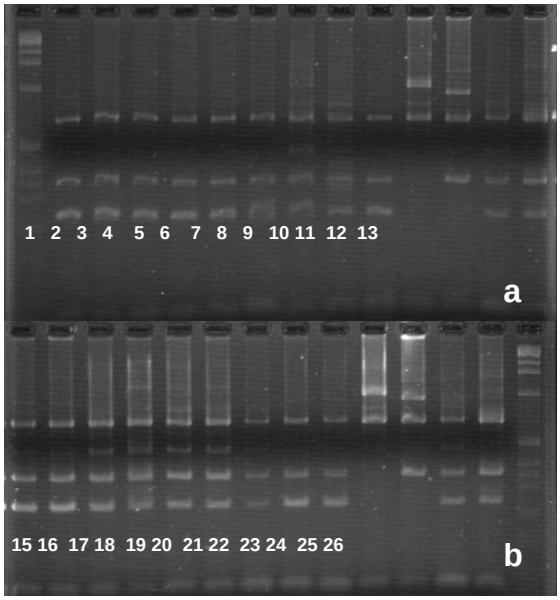

Fig.4 Restriction fragment length polymorphism profiles of VmpA gene sequences amplified from 18 samples and 3 reference controls.
a) From 1 to 13: LU38, LU39, LU40, LU52, LU53, LU54, LU56, LU57, LU68, FD92, FD70, C28, CAM.
b) From 14 to 26: LU70, LU70bis2, LU95bis, MS57, MS58, MS59, MS94, AL1, AL2, FD92, FD70, C28, CAM

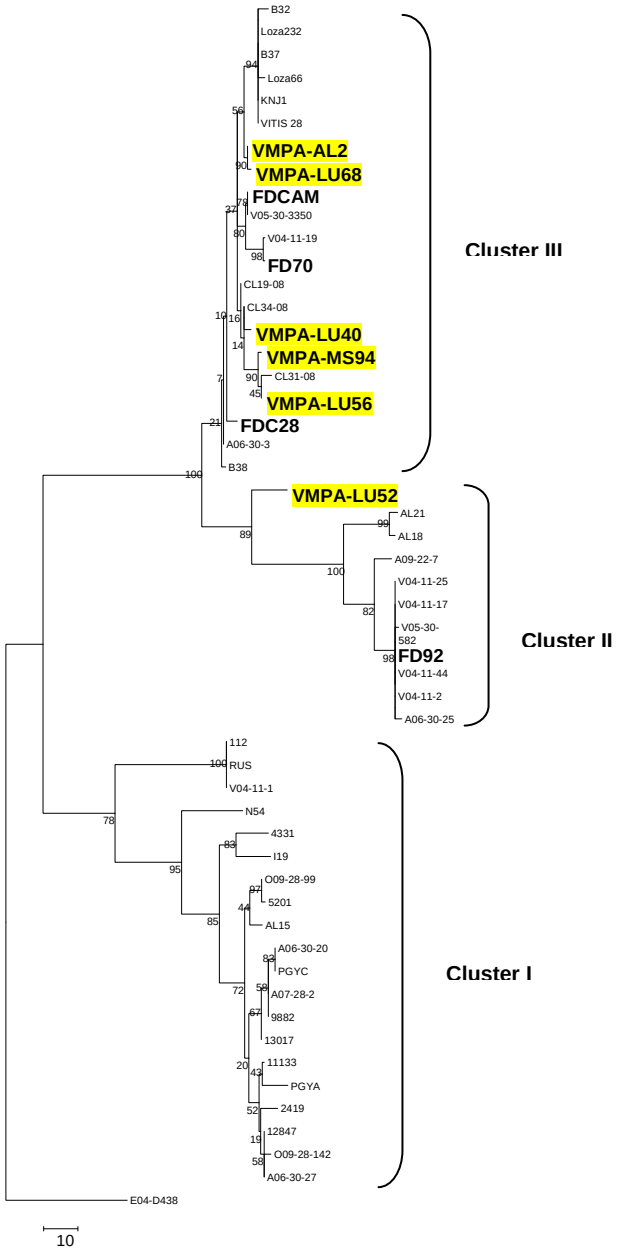

Fig.5 Phylogenetic tree generated using maximum parsimony method with VmpA sequences.

16SrXII-A isolates

SecY: SecY gene was successfully amplified from 24 out of 37 samples (fig. 6 a,b). PCR products from 16 samples, showing the brighter bands on agarole gel, were selected for sequencing with primer pair PosecN2/R3.

Analysis of the 16 SecY sequences proved to be consistent with the Tuf-type classification, in fact Tuf type-a (highlighted in green) and Tuf type-b isolates (highlighted in red) clustered in two different groups (fig. 7).

6 of the 7 Tuf-b isolates shared the same SecY nucleotide sequence, as well as 4 of the 9 Tuf-a isolates.

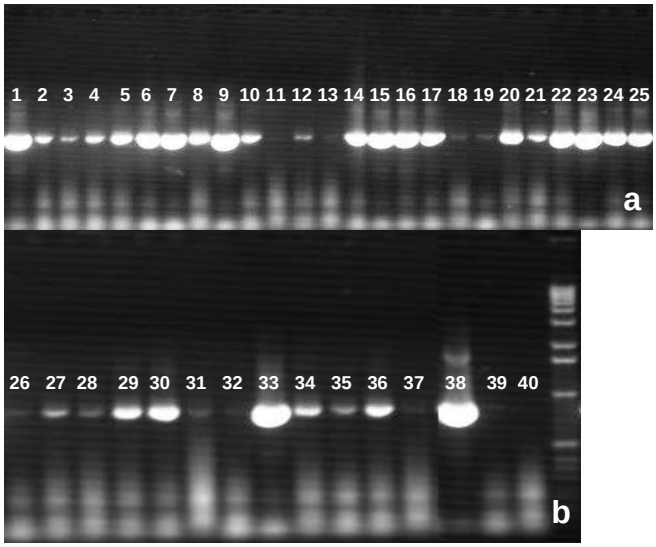

Fig. 6 Amplicons obtained with nested PCR on SecY gene from 37 BN infected samples
a) From 1 to 25: MS62, MS65bis, MS95, MS85bis, MS72, MS71, PI35, PI34, PI33, PI31, PI43, PI39, PI44, PI41, PI38, PI50, PI46, PI47, PI48, LI36, LI50, LI44, LI43, GR26, GR38
b) From 26 to 42: GR24, GR23, GR36, GR37, GR25, GR40, GR29, GR39, LU69, LU73, LU84, MS60, positive control, negative control, H_20.

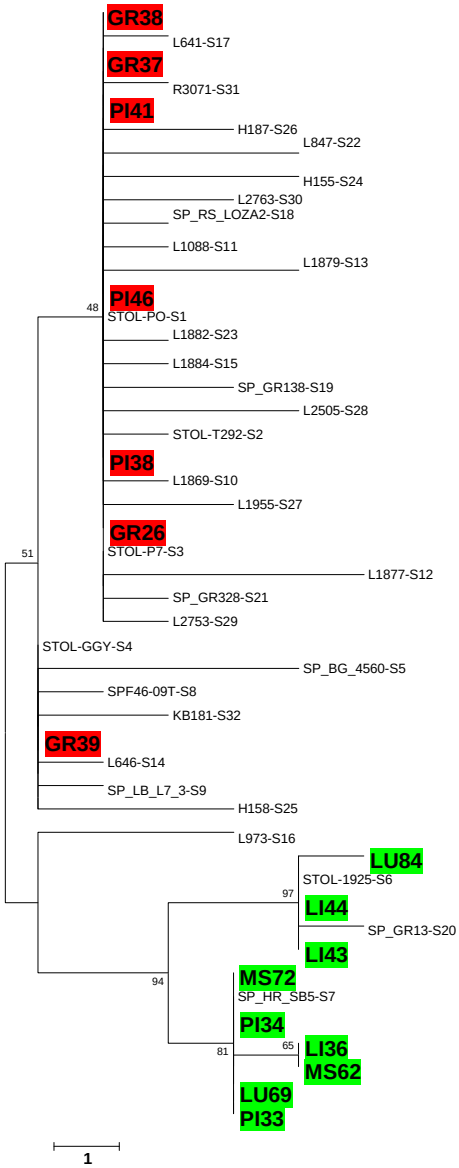

Fig. 7 Phylogenetic tree generated using maximum parsimony method with SecY sequences.

Stamp: Stamp gene was amplified from 30 out of 37 samples. The same 16 samples that were submitted to SecY sequencing were chosen for sequencing of Stamp gene too.
The phylogenetic tree generated with the maximum parsimony method (fig. 8) grouped the isolates in two main clusters comprising, respectively, Tuf-b (in red) and Tuf-a (in green) type isolates.
Sample GR39 presented a different sequence than the remaining tuscan tuf-b isolates, with GR38 showing 1 nucleotide difference compared to isolates GR37, PI41, PI46, PI38 and GR26, which shared the same Stamp nucleotide sequence.
Tuf-a tuscan isolates LI44, LU84 and LI43 proved to have the same sequence, with 1 nucleotide difference compared to LU69, MS72, PI33 and PI34. Isolates MS62 and LI36 showed a different Stamp sequence.

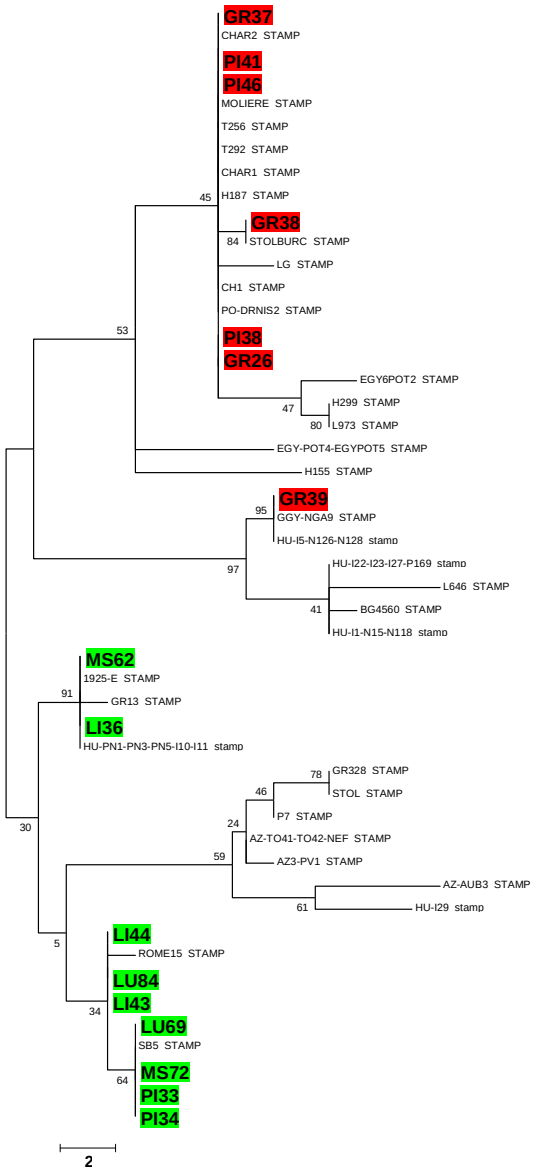

Fig. 8 Phylogenetic tree generated using maximum parsimony method with Stamp sequences.

Vmp1: Nested PCR with primer pairs STOLH10F1/R1 and TYPH10F/R was performed on the 30 samples resulted positive to Stamp. Amplification of Vmp1 gene was not successful, after electrophoresis on agarose gel only 8 out of 30 samples showed very weak bands of the expected size (fig. 9).

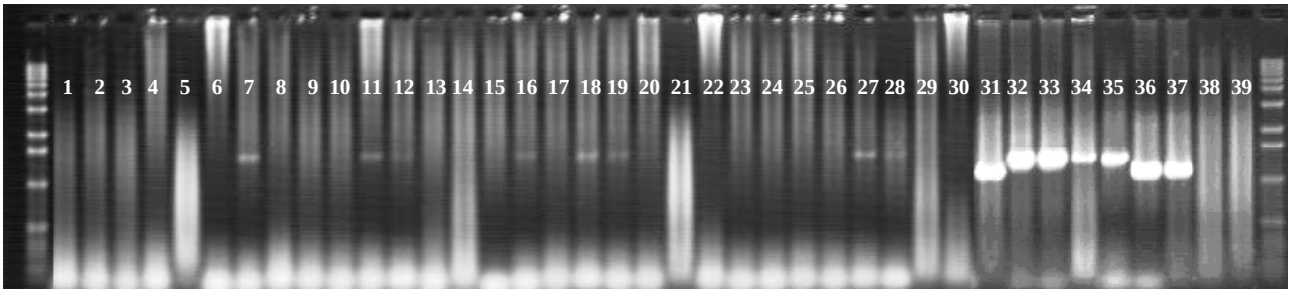

Fig. 9 Amplicons obtained with nested PCR on Vmp1 gene from 30 BN infected samples
From 1 to 30: MS62, MS72, MS71, PI35, PI34, PI33, PI31, PI43, PI39, PI41, PI38, PI50, PI46, PI47 LI36, LI50, LI44, LI43, GR26, GR38, GR23, GR36, GR37, GR25, GR40, GR29, GR39, LU69, LU73, LU84.
From 31 to 37: positive controls
From 38 to 39: Healthy and H_20 controls.

Future collaboration with host institution

The results obtained from this scientific mission allowed beginning the molecular characterization of phytoplasmas infecting vineyards in Tuscany.

Considering DegV and Vmp1 genes were not successfully amplified from a relevant number of samples, it has been planned to repeat the amplification and typing of such genes in our laboratory in Pisa, using different dilutions of extracted DNA and/or using a different Taq Polymerase.

Furthermore, we plan to apply the techniques I learned during this scientific mission to a wider range of phytoplasma isolates from Tuscany and hopefully participate to further and more exhaustive projects about phytoplasma molecular characterization in collaboration with host institution.