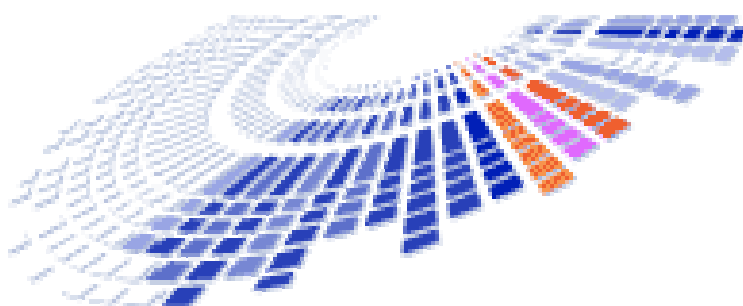


## **NETWORK**

# **Global epidemiology of phytoplasma diseases of economic importance in Southeast Europe**

## **FINAL REPORT**



# PROJECT AT A GLANCE

## **Project title and duration**

Global epidemiology of phytoplasma diseases of economic importance in Southeast Europe.  
(Network PHYTOPLASMA-EPIDEMIO, 9 months starting November 2007)

## **Project Coordinator**

Dr. Xavier Foissac (team INRA-Bordeaux-France)

## **Network objectives**

The network PHYTOPLASMA-EPIDEMIO will coordinate the efforts of plant pathologists, microbiologists and entomologists of Southeast European countries to better monitor phytoplasma strains propagation in grapevine, fruit and vegetables crop through nurseries and insect vectors, at the European scale. The network will promote the use of molecular typing of phytoplasma strains and initiate the development of mitochondrial gene markers for the description of hemipteran vectors at species and ecotype level, to promote phylogeographic studies and describe co-evolution between phytoplasma strains and insect vector ecotypes.

## **Network participants :**

The 13 SEE groups with diverse multidisciplinary phytoplasma expertise that wish to join the network are from 8 eligible countries including Croatia (1), France (2), Germany (2), Greece (2), Hungary (2), Former Yugoslav Republic of Macedonia (1), Romania (1) and Serbia (2) and represent 59 participants including 25 team members under 35 years.

## **Network governance and management**

The network will be run by a management committee consisting of an overall coordinator (Dr. Xavier Foissac, coordinating team) and task leaders. The committee will have the responsibility for administration, network communication, meeting organization and ensuring that working groups remain on schedule to achieve their milestones.

The committee will be responsible of periodic and final reports.

To improve fine monitoring and result dissemination, each Task Leader will transmit at milestone and no later than two month before beginning tasks, timetables describing detailed subtasks with team members and other local partners involvement and budgeting. Task Leader will transmit task reports no later than two months after task completion.

Specific milestones and timetables are included in tasks descriptions. Duration of tasks 2, 3, 4 is from month 1 to month 9, tasks reports will be communicated every 3 months.

## **Networking activity**

Two meetings and a workshop dedicated to molecular typing in addition to bilateral visits will constitute the networking activity. The first meeting will take place during the first meeting of the International Phytoplasma Working Group (IPWG, Bologna, november 2007). Six SEE-eranet phytoplasma working groups corresponding to tasks 2 to 7, will concentrate on three different phytoplasmas of high economical impact.

## **Network tasks**

1. First meeting Bologna
2. Typing of FD and related phytoplasma isolates (groups 16SrV-C and -D)

3. Typing of stolbur phytoplasma isolates (group 16SrXII-A)
  - 3.1 Collection of vegetable, wild plants and grapevine samples
  - 3.2 Collection of cixiids
4. Typing of fruit tree phytoplasma isolates (group 16SrX)
  - 4.1 Collection of stone fruit samples displaying early burst
  - 4.2 Collection of fruit tree psyllids
5. Development of mitochondrial markers for insect typing
6. Database for phytoplasma strains description
7. Workshop and second meeting

### Tasks participants

Teams	Task 1	Task 2	Task 3		Task 4		Task 5	Task 6	Task 7
			Task 3.1	Task 3.2	Task 4.1	Task 4.2			
INRA-Bordeaux-France									
PPI-HAS-Hungary									
IPPE-Zemun-Serbia									
PSI-ISC-Macedonia									
PPL-AUTH-Greece									
LPP-UTH-Volos-Greece									
DBUZ-IPPAF-Croatia									
BBA-Bernkstl-Germany									
ICDPP-Bucharest-RO									
PPDL-EXPERTA-Hungary									
RLP-AIPlanta-Germany									
PERI-Belgrade-Serbia									
INRA-Montpellier-FR									

### Task milestones and deliverables

#### 1. First meeting Bologna

Task leader : X. Foissac (team INRA-Bordeaux-France)

Task Duration : 11-16 november 2007

Task milestones & deliverables:

Planning for the network, working groups organized as well as bilateral visits and exchanges.

List of phytoplasma isolates to be typed.

List of participants established for the six working groups :

#### 2. Typing of FD and related phytoplasma isolates (groups 16SrV-C and -D)

Task leader : S. Malembic-Maher (team INRA-Bordeaux-France)

Task Duration : november 2007 – july 2008

Task milestones & deliverables:

Milestone 1 : final isolate list and markers : december 2007.

Molecular typing of grapevine FD phytoplasma isolates from Serbia, France and other countries if applicable

Molecular typing of alder phytoplasma isolates from all countries involved.

#### 3. Typing of stolbur phytoplasma isolates (group 16SrXII-A)

Task leader : Michael Maixner (team BBA Bernkastel-Germany )

Task Duration : november 2007 – july 2008

Task milestones & deliverables:

Milestone 1 : final isolate list and markers : january 2007.

Milestone 2 : second list of isolates collected in june-july 2008 for molecular typing before end of 2008.

Molecular characterization of stolbur phytoplasma isolates from grapevine, solanaceous crop, lavender, strawberry, insect vectors in eight different countries of Southeast Europe.

Geographical and ecological mapping of stolbur strains in 8 different countries of Southeast Europe.

### **3.1 Collection of vegetable, wild plants and grapevine samples**

SubTask leader : Dijana Škorić (team DBUZ-IPPAF-Croatia)

SubTask Duration : july-august 2008

SubTask milestones & deliverables:

Milestone 1 : Identification of sample collection to be conducted to improve representativity of sample set. May 2008.

New collection of samples displaying stolbur symptoms in solanaceous crop, celery, stawberry, lavender, grapevine, bindweed, nettle and other wild plant hosts during july and august 2008 increasing representativity of variability study.

### **3.2 Collection of cixiids**

SubTask leader : Slobodan Krnjajić (team IPPE-Zemun-Serbia)

SubTask Duration : june-july 2008

SubTask milestones & deliverables:

Milestone 1 : Definition of the collecting strategy, regions and countries, crop or wild environment targetted.

Collection of cixiids (*Hyalesthes obsoletus*, *Reptalus panzeri*, *Pentastiridius* sp., ...) for checking infected status and characterization of stolbur phytoplasma strains proven to be propagated by insect vectors.

New cixiid DNA positive for phytoplasma infection available for molecular typing of stolbur phytoplasma strains proven to be propagated by insect vectors.

## **4. Typing of fruit tree phytoplasma isolates (group 16SrX)**

Task leader : Wolfgang Jarausch (team RLP-AlPlanta-Germany)

Task Duration : november 2007 – july 2008

Task milestones & deliverables:

Milestone 1 : final isolate list and markers : january 2007.

Milestone 2 : second list of isolates collected during winter and spring 2008 for molecular typing during the final training course. may 2008.

Molecular characterization of apple proliferation, pear decline and European stone fruit phytoplasma isolates from orchards in different countries of Southeast Europe as well as from psyllid vectors.

Geographical and ecological mapping of group 16SrX strains in 8 different countries.

### **4.1 Collection of stone fruit samples displaying early burst**

SubTask leader : Sandor Sule (team PPI-HAS-Hungary)

SubTask Duration : january-april 2008

SubTask milestones & deliverables:

Milestone 1 : Identification of sample collection to be conducted to improve representativity of sample set. december 2008.

New isolates for European stone fruit yellows phytoplasmas to be submitted to molecular typing during the workshop in Bordeaux.

#### **4.2 Collection of fruit tree psyllids**

SubTask leader : Gérard Labonne (team INRA-Montpellier-France)

SubTask Duration : march-may 2008

SubTask milestones & deliverables:

Milestone 1 : Definition of the collecting strategy, regions and countries, crop or wild environment targetted. February 2008

DNA from different species of psyllids of different origin. New phytoplasma strain proven to be epidemic available for typing during the workshop in Bordeaux.

#### **5. Development of mitochondrial markers for insect typing**

Task leader : Nicolas Sauvion (team INRA-Montpellier-France)

Task Duration : november 2007 – july 2008

Task milestones & deliverables:

Milestone 1: Mitochondrial markers identified. april 2008.

Development of mitochondrial markers for molecular typing of *Scaphoideus titanus*, *Hyalesthes obsoletus*, *Reptalus panzerii*, *Cacopsylla pyri*, *C. pruni*, *C. melanoneura* and *C. picta*.

First description of mitochondrial haplotypes for these insect vectors.

#### **6. Database for phytoplasma strains description**

Task leader : Florin Oancea (team ICDPP-Bucharest-Romania)

Task Duration : november 2007 – july 2008

Task milestones & deliverables:

Milestone 1 : 2 months after first meeting

Definition of the phytoplasma isolate properties to be described

Milestone 2 : 5 months after first meeting

Definition of the database format and the team and website which will host the database and its online access.

Milestone 3 : 1 month before the second meeting in Bordeaux the database is functional.

Milestone 4 : Uploading of isolate description during the workshop in Bordeaux.

A first version of the database to describe phytoplasma strains.

#### **7. Workshop and second meeting**

Task leader : Xavier Foissac (team INRA-Bordeaux-France)

Task Duration : 22-28 june 2008

Task milestones & deliverables:

Milestone 1 : Participant list available february 2008

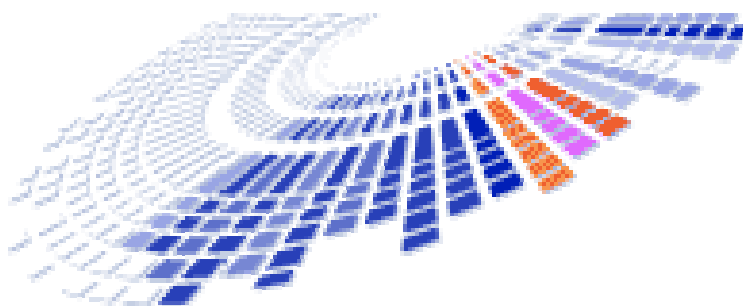
Milestone 2 : Tentative programme for the workshop proposed. March 2008

Milestone 3 : Final programme made available with all protocoles and the list of phytoplasma and insect isolates to be tested during the workshop. April 2008.

Milestone 4 : Final programme for the two days meeting (scientific reports, prospective for FP7). May 2008.

## **NETWORK**

# **GLOBAL EPIDEMIOLOGY OF PHYTOPLASMA DISEASES OF ECONOMIC IMPORTANCE IN SOUTHEAST EUROPE**



## **FIRST MEETING AND PLANNING MEETING**

**11 & 16 November 2007**

**Holiday Inn Hotel – Bologna  
& University of Bologna**

# LIST OF PARTICIPANTS

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Xavier Foissac	FRANCE-INRA.Bordeaux	foissac@bordeaux.inra.fr

<p align="center"><b>First meeting (1 h 30)    Sunday 11 Nov. 2007    17h30-19h</b>  <b>Holiday Inn hotel</b></p>
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Presentation of the network objectives and Consortium. Presentation of tasks – X. Foissac 10 min

Roundtable presentation of team members by team leaders – 20 min

Presentation of tasks by task leaders for each working group.

Setting-up of the list of WG participants – 30 min

Proposition of bilateral visits for team 3 (IPPE- Serbia), team 5 (PPL-AUTH Greece), team 6 (LPP-UTH Greece) and team 7 (DBUZ-IPPAF Croatia). 10 min

Open discussion 15 min

Conclusion and future milestones – X. Foissac 5 min

<p align="center"><b>Second meeting (2 h30)            Friday 16 Nov. 2007    8h30-11h00</b>  <b>University of Bologna</b></p>
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Presentation of the programme for second meeting

Setting up of WG mailing list and network mailing list    **10 min**

For WG1, WG2, WG3 andWG5 20 minutes each

**Presentation by task leaders – discussion with WG participants**

Presentation of task objectives, milestones

and provisional lists of isolates or insect populations 10 min

Strategy for selecting isolates or completing the final list - 5 min

Choice for markers - work plan for typing 5 min

For WG 4, 20 minutes

**Presentation by task leaders – discussion with WG participants**

Task objectives and milestones 5 min

Discussion about the phytoplasma isolate properties to be described in the database 5 min

Form of the database, strategy for information upload and update (curators ?), work plan 10 min

For WG 6 25 minutes

Practical workshop – identify needs 5 min

Discussion about possible programs 10 min

Provisional list of participants, date & organizing committee 10 min

<p align="center"><b>Planning for one week SEE-ERANET bilateral visits</b></p>
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<b>Person</b>	<b>host institution</b>	<b>date</b>
Martina Seruga Music	INRA-Bordeaux	February 2008
Jelena Jovic	INRA-Bordeaux	May 2008
Asimina Katsiani	INRA-Bordeaux	June 2008
Evangelos Vellios	GERMANY-RLP.AIPlanta	July 2008



**Working Group 1: Phytoplasmas of the 16SrV group**

- Typing by RFLP on map and secY loci for groupV phytoplasma detected on grapevine
- Collection of alders in Republic of Serbia
- Sequencing of map gene for isolates detected in alders

**Working group 2 : Stolbur phytoplasmas (group 16Sr-XII-A)**

- As the project timetable does not overlap august and September which correspond to the best moment to see stolbur symptoms, decision is made to set up a list of available frozen plant DNA isolates concentrating mainly grape, bindweed, nettle, solanaceous crop, corn.
- The team from IPPE-Serbia produce and communicate a document containing protocols for collecting and provide pictures in order to recognize the most common European cixids, most of them vectors of stolbur phytoplasmas.
- Typing markers will be Tuf-RFLP with HpaII, secY sequencing and VmpI RFLP with RsaI or vmpI sequencing.
- INRA-Bordeaux has to provide ASAp the protocols for amplifying secY and VmpI (former stolIH10 gene)

**Working group 3 : fruit tree phytoplasmas (group 16Sr-X)**

- According to W. Jarausch it is still time to sample for Apple proliferation
- Croatian team will provide pear decline sample
- Most of the samples currently being typed by sequencing 4 makers (MLST) by at INRA-Bordeaux are from France, Italy, Germany, Spain, Croatia.
- For psyllids B. Jarausch and N. Sauvion would like the participants to collect 5 insects from 5 sites per countries to have a first evaluation of taxonomic status of the insects and infection status. Samples should be individually stored in dry tubes or in absolute ethanol (no 70% ethanol) and send to Germany on dry ice for identification.

**Working group 4 : database**

- An agreement is made of an excel format for each phytoplasma group and will be send to participants by W. Jarausch.
  - A specific SEE-ERANET code is designed A= AP, E= ESFY, P=PD, F = grapevine group V, S=Stolbur, X= alder isolates. P =plant sample, I= Insect sample
- Two letter for the country-two digit for the region-three digit as sample number in this region. The original laboratory sample id number will be kept in an independent column in the excel file. Example SP-F-33-001 is the first plant stolbur sample from Gironde France

**Working group 5: Insect typing**

- Mitochondrial typing preferred because of the multiple copies, no introns, better resolution. Cytochrome Oxydase I region targeted.

**Working group 6 :**

- the practical workshop and final meeting will be held at INRA-Bordeaux on 23-27 June.

## **Task 2 : Typing of Flavescence dorée and related phytoplasma isolates (groups 16SrV-C and -D)**

**Coordinator** : Sylvie Malembic-Maher, belonging to team INRA-Bordeaux-France

**The context** : The Flavescence dorée (FD) of grapevine is a quarantine disease caused by phytoplasmas of the 16SrV group (Boudon-Padieu, 2002). Transmitted by a leafhopper of North American origin (*Scaphoideus titanus*) (Schvester *et al.*, 1963), it is spread in the vineyards of South-western Europe and has been recently reported in the Balkans (Duduk *et al.*, 2004). The genetically related alder phytoplasmas (AldYp) are very common in Europe. They are transmitted by the leafhopper *Oncopsis alni* which can occasionally inoculate them to grapevine, leading to Palatinate Grapevine Yellows (PGY) disease (Maixner & Reinert, 1999; Maixner *et al.*, 2000). According to recent findings, these phytoplasmas could constitute a wild reservoir of FD (Angelini *et al.*, 2003; Arnaud *et al.*, 2007).

**Objectives** : To gain ground on the genetic and biological relationships between AldY and FD phytoplasmas, we will study their diversity by PCR-RFLP and MLST typing of infected plant and insect DNA extracts collected in the different countries involved in the program.

**Methodology** : DNA extracts from the established list will be submitted to a real time PCR detection test to verify their infection status. PCR-RFLP will be performed on the *map* gene to differentiate FD subgroups. Finally, a multilocus sequence typing of non ribosomal genes (*rpsC*, *secY*, *map* and *degV*) will be realised according to Arnaud *et al.*, 2007.

**Task Input** : DNA extracts from infected grapevine, alders and leafhopper vectors.

**Result, milestones** : 1. Final isolate list and markers, december 2007. 2. Molecular typing of grapevine FD phytoplasma isolates from Serbia, France and other countries if applicable. 3. Molecular typing of alder phytoplasma isolates from all countries involved.

### **I. Constitution of the list of samples**

In September 2008, the following call was sent to all the participants in order to constitute the list of DNA samples :

- Grapevine and/or *S. titanus* samples : countries where FD is recorded (Serbia and France) should compile infected samples representative of the main focal points on their territory.

Twenty to 30 samples by country should be enough. Countries where FD is not recorded could eventually compile other grapevine yellows phytoplasma isolates already identified as 16SrV.

- Alder samples : countries having FD should collect symptomatic alder samples in a vine area where FD outbreaks have been identified, in a vine area known free of FD and in a vine free area. Countries without FD, if interested, could collect symptomatic alders in one area (with or without vines). Ten samples from each area should be sufficient.

During the first meeting in Bologna, 5 teams confirmed their participation in the task 2 : INRA-Bordeaux – France, IPPE- Serbia, BBA- Germany, EXPERTA LTD – Hungary, PERI – Serbia. IPPE and INRA Bordeaux teams transmitted a first list of collected FD phytoplasma (FDp) isolates from grapevine and *S. titanus*, and AldY phytoplasma (AldYp) isolates from alder.

- BBA team declared his intention to transmit a list of collected PGY phytoplasma (PGYp) isolates from grapevine and AldYp isolates from alder and *O. alni*.
- PERI team declared his intention to transmit a list of collected FDp isolates from grapevine.
- EXPERTA team declared his intention to collect symptomatic alder samples.

Before the end of December, the final lists tables in the annex were established. The lists were presented according to the format collectively established during the Bologna meeting. A total of 132 isolates were collected : 57 from alder (AldYp), 50 from grapevine (FDp and PGYp), 18 from *O. alni* (AldYp) and 7 from *S. titanus* (FDp). Photos of some samples are presented in Fig 1. A summary of the collected isolates is also presented in Table 1.

## **II. Detection of the phytoplasmas in the samples**

Detection of 16SrV phytoplasma in the samples had already been done by the different teams by single or nested-PCR with group V specific primers. The primers and the results of the PCR are detailed in annex's tables. Thus, we decided not to check the infection status by real-time PCR and to directly realise the typing. A practical session entitled “Detection of Flavescente dorée and Bois noir phytoplasmas in grapevine by a triplex real-time PCR” was organised during the workshop (see task 7 report).

## **III. Typing of the phytoplasmas**

In a first step, the typing of every isolates was performed by sequencing of the *map* gene as described in Arnaud *et al.*, 2007. In a second step, some isolates were selected for the sequence typing with a second marker *uvrB-degV* gene (Arnaud *et al.* 2007). IPPE team

realized the typing of their own isolates and INRA Bordeaux team characterized the rest of the isolates.

The *map* gene (800 bp) was amplified by nested PCR for 126 isolates. After sequencing on one strand with MAPR2 primer, the chromatograms of 116 isolates were fully exploitable on 674 bp. Some isolates' chromatograms revealed sequences ambiguities (superposition of nucleotide pics) which were detected for one up to ten positions. This indicates a mix of different phytoplasma populations in the same sample. "Mixed" infections were detected, in 1/18 *O. alni* and in 30/56 alders but were not detected in grapevine and *S. titanus* (see Table 1). For the mixed infections, the *map* gene was re-amplified by single PCR with a *taq* proof-reading enzyme (30 cycles with the FD9F6 and MAPR2 primers), cloned in the pGEMt Easy plasmid and 4 clones were sequenced (named A, B, C and D). The cloning was performed at INRA Bordeaux during the bilateral visit of Jelena Jovic from IPPE team-Serbia (from the 25<sup>th</sup> to 31<sup>st</sup> of may) . She was trained to the cloning techniques and we were able to clone 8 French and 3 German alder isolates (Table 1 and annex). Cloning of the Serbian isolates is in course. Phylogenetic analyses were performed on a total of 117 sequences plus 19 sequences of reference strains (Arnaud *et al.* 2007). A phylogenetic tree is presented Fig. 2. Forty six different genotypes were identified : 3 in *S. titanus*, 7 in *O. alni*, 11 in grapevine and 37 in alder. Analyses confirmed that grapevine and alder phytoplasmas have a common phylogenetic origin. Twelve genetic clusters could be described. Three clusters containing FDp isolates were named FD1, 2 and 3. FD 1 was strictly French, FD2 was French and Italian and FD3 was Serbian and Italian. No German isolates were found in these clusters. French AldYp isolates with exactly the same genotype as FD isolates were found in the FD1 cluster. French and Serbian AldYp isolates presented 3 and 4 SNP's respectively with the FD isolates from the cluster FD2. There were no AldYp isolates genetically close to the FD3 cluster. Nine genetic clusters containing AldYp and PGYp isolates were named AldY 1 to 9. They contained German, Hungarian, Serbian and French isolates.

The *uvrB-degV* gene was amplified and sequenced for 8 grapevine isolates (5 from Germany and 3 from Serbia) (See Table 1 and annex). Two different genotypes were identified in Germany and 2 different ones in Serbia. This is the first time that different genotypes are identified in grapevines from Serbia. A phylogenetic tree was realized, including the sequence of 23 reference strains (Fig. 3).

#### **IV. Conclusion and perspectives**

We could collect and molecularly characterize FDp isolates from different viticultural areas in Serbia and France, and AldYp isolates from viticultural and non-viticultural areas in Serbia, France, Germany and Hungary. The sequence typing of the *map* gene showed that:

- The phytoplasmas from alder and grapevine have a common phylogenetic origin.
- Some AldYp genotypes are identical (or very close) to FDp genotypes in FD-contaminated and non-contaminated areas.
- In alders, there is an important diversity of isolates and mixed infections are frequent whereas in grapevine the diversity is poorer and we could not find any mixed infection.

These results strengthen our hypothesis that AldY constitutes the original reservoir of FD in Europe with a low frequency of phytoplasma exchange between alder and grapevine. These results led to a first publication (Cvrković *et al.*, 2008) and to two presentations at scientific congress (Malembic-Maher *et al.*, 2007; Malembic-Maher *et al.*, 2008).

In order to confirm our hypothesis, we will realize the molecular typing of alder and grapevine isolates from northern Italy where FD is present. We will also evaluate the ability of *S. titanus* to transmit AldYp and PGYp isolates and especially isolates which have the same genotypes as FDp isolates.

**Angelini, E., Negrisol, E., Clair, D., Borgo, M. & Boudon-Padieu, E. (2003).** Phylogenetic relationships among Flavescence dorée strains and related phytoplasmas determined by heteroduplex mobility assay and sequence of ribosomal and non-ribosomal DNA. *Plant Pathol* **52**, 663-672.

**Arnaud, G., Malembic-Maher, S., Salar, P., Bonnet, P., Maixner, M., Marcone, C., Boudon-Padieu, E. & Foissac, X. (2007).** Multilocus sequence typing confirms the close genetic inter-relatedness between three distinct flavescence dorée phytoplasma strain clusters and group 16SrV phytoplasmas infecting grapevine and alder in Europe. *Applied and Environmental Microbiology* **73**, 4001-4010.

**Boudon Padieu, E. (2002).** Flavescence dorée of the grapevine: knowledge and new developments in epidemiology, etiology and diagnosis. *ATTI Giornate Fitopatologiche* **1**, 15-34.

**Cvrković, T., Jović, J., Mitrović, M., Petrović, A., Krnjajić, S., Malembic-Maher, S. & Toševski, I. (2008).** First report of alder yellows phytoplasma on common alder (*Alnus glutinosa*) in Serbia. *Plant Pathology* **57**, 773.

**Duduk, B., Botti, S., Ivanovic, M., Krstic, B., Dukic, N. & Bertaccini, A. (2004).** Identification of phytoplasmas associated with grapevine yellows in Serbia. *Journal of Phytopathology* **152**, 575-579.

**Maixner, M. & Reinert, W. (1999).** *Oncopsis alni* (Schrank) (*Auchenorrhyncha: Cicadellidae*) as a vector of the alder yellows phytoplasma of *Alnus glutinosa* (L.) Gaertn. *European Journal of Plant Pathology* **105**, 87-94.

**Maixner, M., Reinert, W. & Darimont, H. (2000).** Transmission of grapevine yellows by *Oncopsis alni* (Schrank) (*Auchenorrhyncha: Macropsinae*). *Vitis* **39**, 83-84.

**Malembic-Maher, S., Salar, P., Vergnes, D. & Foissac, X. (2007).** Detection and diversity of "flavescence dorée" related phytoplasmas in alders surrounding infected vineyards in Aquitaine - France. First International Phytoplasma Working Group meeting, November 12-15 2007, Bologna Italy. *Bulletin of Insectology* **60**, 329-330.

**Malembic-Maher, S., Cvrkovic, T., Salar, P., Jović, J., Mitrović, M., Petrović, A., Krnjajić, S., Tosevski, I. & Foissac, X. (2008).** Looking for genotypes related to the grapevine Flavescence dorée

phytoplasma among phytoplasmas infecting alders in France and in Serbia. IOM Congress. Tianjin, China.

**Schvester, D., Carle, P. & Moutous, G. (1963).** Transmission de la flavescence dorée de la vigne par *Scaphoideus littoralis* Ball. *Ann Epiphyties* **14**, 175-198.

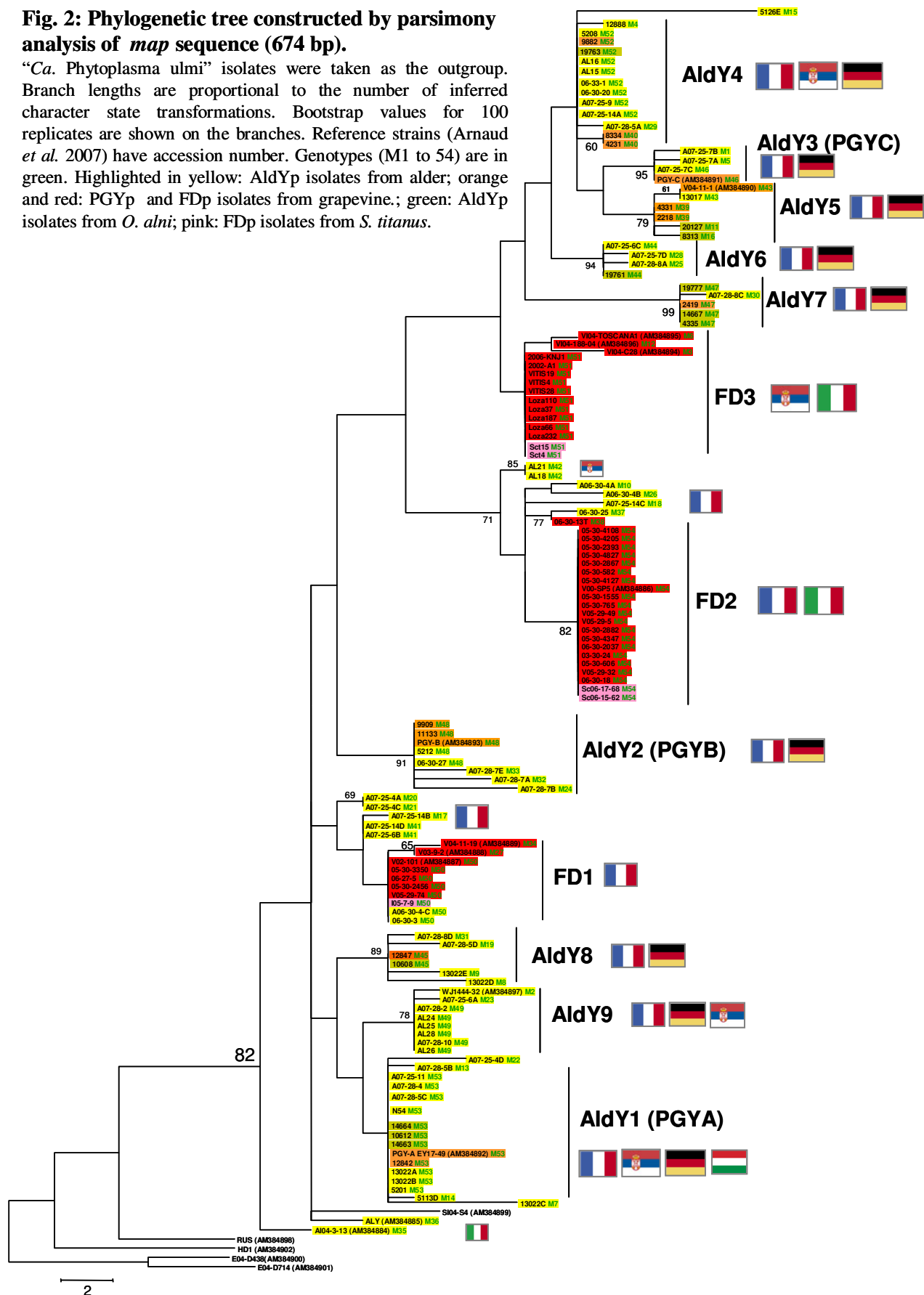




**Fig. 1 : Photos of some collected samples:** **A.** Grapevine infected by Flavescence dorée phytoplasma in France. **B.** Grapevine infected by Palatinate grapevine yellows phytoplasma in Germany. **C.** Alder infected by Alder Yellows phytoplasma. **D.** *Scaphoideus titanus* **E.** *Oncopsis alni*.

**Fig. 2: Phylogenetic tree constructed by parsimony analysis of *map* sequence (674 bp).**

“*Ca. Phytoplasma ulmi*” isolates were taken as the outgroup. Branch lengths are proportional to the number of inferred character state transformations. Bootstrap values for 100 replicates are shown on the branches. Reference strains (Arnaud *et al.* 2007) have accession number. Genotypes (M1 to 54) are in green. Highlighted in yellow: AldYp isolates from alder; orange and red: PGYp and FDp isolates from grapevine.; green: AldYp isolates from *O. alni*; pink: FDp isolates from *S. titanus*.





“*Ca. Phytoplasma ulmi*” isolates were taken as the outgroup. Branch lengths are proportional to the number of inferred character state transformations. Bootstrap values for 500 replicates are shown on the branches. Reference strains (Arnaud et al. 2007) have accession numbers. Genotypes (U1 to U16) are in green. Highlighted in yellow: AldYp isolates from alder; orange and red: PGYp and FDP isolates from grapevine.



**Table 1 : Results of *map* and *uvrB-degV* genes sequence typing from all the 16SrV collected isolates.**

Gene	Sample	Country	PCR Positive Samples analysed	Low quality rejected sequence	Single infection	Mixed infection	Cloned From mixed infection	Genotypes
<i>map</i>	<i>Alnus sp.</i>	France	30	3	10	17	8	M1, M5, M10, M13, M17, M18, M19, M20, M21, M22, M23, M24, M25, M26, M28, M29, M30, M31, M32, M33, M37, M41, M44, M46, M48, M49, M50, M52, M53
		Germany	10	0	4	6	3	M4, M7, M8, M9, M14, M15, M43, M48, M53
		Hungary	2	1	1	0	0	M53
		Serbia	15	0	8	7	0	M42, M49, M52
	<i>Vitis sp.</i>	France	23	0	23	0	0	M38, M50, M54
		Germany	12	1	11	0	0	M39, M40, M45, M47, M48, M52, M53
		Serbia	12	2	10	0	0	M51
	<i>Oncopsis alni</i>	Germany	17	3	13	1	0	M11, M16, M44, M45, M47, M52, M53
	<i>Scaphoideus titanus</i>	France	3	0	3	0	0	M50, M54
		Serbia	2	0	2	0	0	M51
		<b>TOTAL</b>	<b>126</b>	<b>10</b>	<b>85</b>	<b>31</b>	<b>11</b>	<b>46 genotypes</b>
<i>uvrB-degV</i>	<i>Vitis sp.</i>	France	0	0	0	0	0	
		Germany	5	1	4	0	0	U7, U11
		Serbia	2	0	2	0	0	U1, U14
		<b>TOTAL</b>	<b>7</b>	<b>1</b>	<b>6</b>	<b>0</b>	<b>0</b>	<b>4 genotypes</b>

### **Task 3 : Typing of stolbur phytoplasma isolates (group 16SrXII-A)**

**Coordinator** : Michael Maixner, belonging to team BBA Bernkastle-Kues

**The context** : The stolbur phytoplasma (group 16SrXII-A) is an European endemic phytoplasma of wild compartment origin and is transmitted from bindweed, nettle and lavender to grapevine, maize, solanaceous crop (tomato, pepper, potato, eggplant), strawberry and sugarbeet by polyphagous planthoppers of the Cixiidae family. Some stolbur phytoplasma strains recently appeared to be associated with specific insect vector ecotype which could indicate some strains specialization (Langer and Maixner, 2004).

**Objectives** : Establish a list of stolbur DNA isolates collected on plant hosts or insect vectors. Evaluate the genetic diversity of stolbur phytoplasma at the European geographical scale.

**Methodology** : DNA extracts from the established list will be submitted to stolbur-specific PCR detection test to verify their infection status. Genotyping of the stolbur isolates by using genotyping tools based either on RFLP patterns of the variable gene *vmp1* encoding a putative membrane protein and the house-keeping gene *tuf* or by sequencing of the *secY* gene.

**Task Input** : DNA extracts from infected grapevine, wild plant and planthopper vectors.

**Result, milestones** : 1. Final isolate list and markers, january 2008. 2. Molecular typing of stolbur plant isolates. June 2008. 3. Molecular typing of stolbur insect isolates. July 2008.

#### **1. Constitution of the list of samples**

Nine partners participated to the setting up of the collection of DNA isolates namely DBUZ-IPPAF (Croatia), INRA-Bordeaux (France), BBA-Bks-UnivMainz (Germany), LPP-UTH (Greece), PPD-L-Experta (Hungary), PSI-ISC (Macedonia), IPPE (Serbia), PERI-Belgrade

(Serbia), UTH (Greece). Altogether 311 plants samples including 265 grapevine samples as well as 14 bindweed and stinging nettle samples and fifteen samples of other plant species were proposed to be included in the genotyping study.

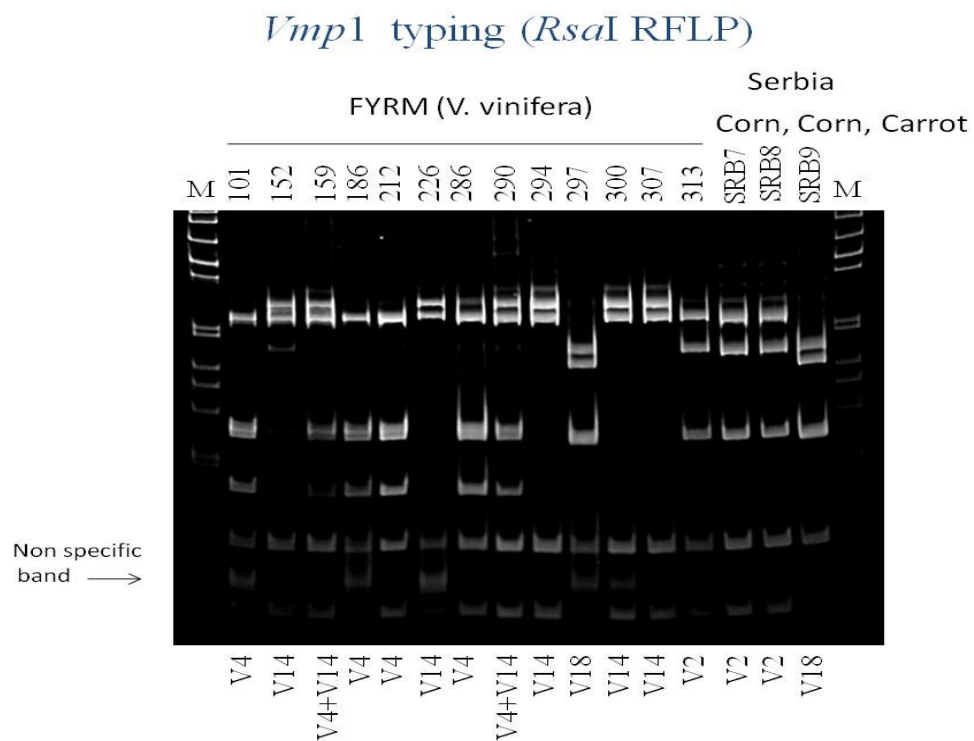
For the insect vectors, 174 DNA extracts were proposed including 170 *Hyalesthes obsoletus* from Germany, Croatia and Serbia.

## **2. Plant Genotyping :**

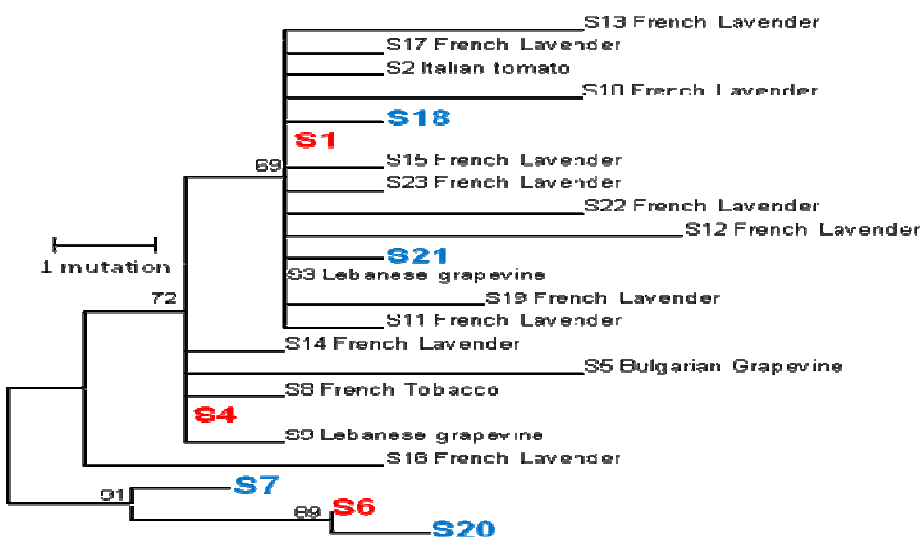
Hundred and twenty seven samples were typed by *Hpa*II-RFLP of *tuf*-PCR : 79 samples were of *tuf*II genotypes while 43 samples were of *tuf*I and 5 of *tuf*III genotypes. This may indicate that in our collection two third of the isolate were coming from bindweed reservoir. In Balkan countries genotype *tuf*II was largely dominating, while in France and Germany the ratio between the two genotypes was balanced.

According to *vmp*1 *Rsa*I-PCR-RFLP, 210 samples could be examined. Fourteen different RFLP patterns could be evidenced. Pattern V1 (73 isolates) and pattern V4 (45 isolates) were predominating. The V1 genotype appeared specific of France and Germany, while the genotype V4 was present in all countries except Hungary. Pattern V2 accounted for 28 and V3 for 18 isolates. The latter was absent from Germany. V18 and V14 represented 15 and 11 isolates respectively and were specifically abundant in the Balkan countries. Genotypes V6, V7, V8, V12 and V19 were only detected in one or two French grapevine samples, whereas genotype V13 was only detected in one infected grapevine in Croatia and V15 in three samples from Germany. Two mixed infection of V2:V18 genotypes were detected in Croatian grapevines, one mixed infection V14/V2 in a Serbian grapevine and two mixed infection of V4:V14 genotypes in two Macedonian grapevines. In Germany, mixed infection of V1:V4 was found in one grapevine and one bindweed and V1:V2 was detected in a grapevine. As an example results obtained for some samples from the Macedonia and Serbia are illustrated in **Figure 1**.

*SecY* sequence genotypes were obtained for 138 isolates and 7 different genotypes were distinguished (see **Figure 2**). Most of the stolbur genotypes belonged to the S6 (56 isolates), the S1 (44 samples) and the S4 (26 samples) genotypes. Four genotypes, namely S18 (4 samples from Serbia, FYRM, Hungary), S7 (3 samples from Croatia), S20 (1 sample from Greece) and S21 (1 sample from Greece) were only detected in samples from Balkan.



**Figure 1:** Electrophoretic analysis of *RsaI* restriction product of *vmp1* PCR on 8 % polyacrylamide gel. The genotypes V are indicated below the figure.



**Figure 2:** phylogenetic analysis of *secY* genotypes detected in plant samples of the SEE-ERANET consortium. Genotypes in red are the most abundant genotypes. Genotypes in blue colour represent minor genotypes detected in Balkan.

### **3. Insect Genotyping :**

Hundred and sixty one insects were typed by *HpaII*-RFLP of *tuf*-PCR : 81 samples were of *tufII* genotypes while 77 samples were of *tufI* and 6 of *tufIII* genotypes. The genotype *tufII* was the only detected in Serbian insects.

According to *vmp1* *RsaI*-PCR-RFLP, 133 samples could be examined. Eight different RFLP patterns could be evidenced. Patterns V1 (64 insects) and pattern V4 (22 insects) were predominating. Patterns V2 was detected in 11 insects whereas pattern V14 was detected in 2 Serbian insects. Four other pattern, namely V3, V12, V15 and V16 were only detected in one or two insect samples. Eight mixed infections of V1:V2 and three of V1:V4 were found.

*SecY* sequence genotypes were obtained for 18 insect isolates and only the three main genotypes were detected, namely S6 (7 insects), the S1 (5 samples) and the S4 (4 insects) genotypes. Two mixed infection between S1 and S4 genotypes were also evidenced.

# Global epidemiology of phytoplasma diseases of economic importance in Southeast Europe

Task 3. Typing of stolbur phytoplasma isolates (group 16SrXII-A)

Subtask 3.1. Collection of vegetable, wild plants and grapevine  
samples

Dijana Škorić (team DBUZ-IPPAF-Croatia)

Zagreb, May – June, 2008

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# 1. Background

## 1.1. Economical importance of stolbur phytoplasmoses in Southeastern Europe

Phytoplasmoses associated with the agents from the ribosomal subgroup 16SrXII-A have been increasingly important in the countries of Southeastern Europe. Different names have been attributed to them (stolbur, bois noir, legno nero, Schwarzholzkrankheit, Vergilbungskrankheit) depending on the country of occurrence and cultivated plants affected. Early reports drew attention to stolbur “virus” as a serious disease of vegetables (Panjan 1957, Panjan *et al.* 1970) especially potato, tomato and pepper (Aleksić *et al.* 1969) or, generally speaking, those from the *Solanaceae* family. The members of this family also include common weeds that can be found at the borders of the vegetable plots or in and around vineyards. The importance of weeds as alternative stolbur hosts is crucial in the stolbur epidemiological studies and the disease management. This is especially significant for grapevine as a woody perennial stolbur host having long-term impact in its agro-ecosystem. Even though, Flavescence Dorée had been pointed out in the literature as the most serious phytoplasma threatening the European viticulture and the importance of stolbur was thought to be less important, probably given its endemic nature, the epidemics of grapevine yellows (GY) attributable to stolbur has increased over the last 10 years. The problem of simultaneous stolbur outbreaks in different European regions, as well as other open questions regarding the epidemiology pertinent to this network, were summarized in the opening lecture of the GY session at the XV. ICVG meeting (Maixner 2006).

## 1.2. Epidemiological considerations

So far, we have probably the most epidemiological data from stolbur infected German vineyards where the roles of pathosystem’s weed members *Urtica dioica* and *Convolvulus arvensis* have been investigated (Langer and Maixner, 2004). *Calystegia sepium*, another weed from the *Convolvulaceae* family, may also play a role in the distribution of VK-II and VK-III types of stolbur. The body of knowledge about alternative phytoplasma hosts is increasing. Various research groups working on samples from their own countries or in collaboration with foreign scientist have pinpointed other weed species potentially important in phytoplasma epidemiology. Since common vineyard weeds like *Cirsium arvense*, *Taraxacum officinale*, *Plantago* sp., *Medicago sativa*, and even *Polygonum lapathifolium* and *Datura stramonium* continuously appear in reports as stolbur hosts (First International

Phytoplasmaologists Working Group Meeting 2007, Extended Abstracts of the XV. ICVG Meeting 2006, Atti di 3. incontro nazionale sulle malattie da fitoplasmi 2005, Palermo *et al.* 2004, Škorić *et al.* 1998, Panjan 1957) it would be worthwhile to investigate their role in the GY epidemiology more closely.

Shrubs and trees hosts growing as bordering plants (*Sambucus nigra*, *Prunus spinosa*, *Rubus* spp.) should also be considered in global epidemiology investigations because they are perennials whose fixed spatio-temporal position in the ecosystem may influence the course of the disease. Moreover, some of them have already been found to harbour stolbur phytoplasmas.

The fruit and vegetable, especially the staple food plants, production in the countries involved in this network have also suffered serious losses due to stolbur epidemics. While the problem is obviously perpetuating in solanaceous crops and maize from the 1950s in the Balkan area (Panjan 1957, Jović *et al.* 2007, Avramov *et al.* 2008), it has become evident in the last 5-10 years in parsley, celery, carrot, lettuce, and strawberry. The recent study of maize redness disease in Serbia is a good example of early epidemiological study with implications for more efficient disease control (Jović *et al.* 2007).

Most of the countries involved in this networking effort are endowed with some variant of continental climate and, so far, most of the epidemiological data on stolbur come from these types of ecosystems. However, the stolbur-type phytoplasmoses affect cultivated plants in the Mediterranean area with consequences that are especially important in lavender and grapevine production. Even though some of the vectors and herbaceous hosts are found in the Mediterranean vineyards and fields as well as in those of central and eastern Europe, there is not enough information on this type of agro-ecosystems and phytoplasma alternative weed hosts therein.

## 2. Subtask Aim

This subtask aim is to increase the sample representativity of stolbur phytoplasma variability study regarding the grapevine samples, vegetables and wild plants with special regard to ones showing potential phytoplasmoses symptoms.

### 3. Assessment of the type of stolbur isolate distribution in the database

So far the database of plants available for the stolbur phytoplasmas encompasses 289 samples from 5 countries of which about 87% (251 samples) is from grapevine. Since the project is in its final phase, this number is growing and the rough assessment given here will surely change shortly. French and Macedonian stolbur isolates are exclusively from grapevine, while other groups have a proportion of samples from other hosts. These include herbaceous specimens of indigenous flora that grow in or around vineyards as weeds or cultivated plants like corn (8 samples), potato (1 sample), carrot (1 sample) and a peach (1 sample). The latter may not be relevant to the stolbur epidemiology type of study although it reflects an ancient practice in some of the countries (Croatia, Germany, Serbia, others?) of growing a peach from a seedling (so called vineyard peaches) amidst grapevines. This practice exists in some Mediterranean vineyards but in these agro-ecosystems figs are sometimes cultivated in or close to grapevines. The proportion of plant samples other than grapevine is roughly about 20% for the teams that counted them in, but the overall representativity in the plant stolbur database is only 8.3% for weeds and about 3.5% for vegetables. The samples from the Mediterranean area are either not represented yet (Greece), represented only as grapevine samples (France) and scarcely represented (Croatia). No weed or vegetable stolbur isolates have been listed from the Mediterranean yet. The Greek colleagues have announced input of the isolates from tomato.

Vegetable crop isolates have been listed mostly from Serbia (corn, carrot) and weeds have been systemically investigated only in Germany. Lists from other countries probably represent certain focal points, but better representation is needed for good quality epidemiological studies.

### 4. Considerations for plant sample representativity improvement

#### 4.1. Weed sampling

Due to limited time and resources within these networking activities, it would be prudent to sample in well known agro-ecosystems of high interest taking into account specific factors like: climate, relief, natural pytocenoses and factors influencing them. The knowledge of feeding preferences of potential and well known insect vectors is important. The relative abundancy of weeds should also be considered. Weeds are often asymptomatic phytoplasma

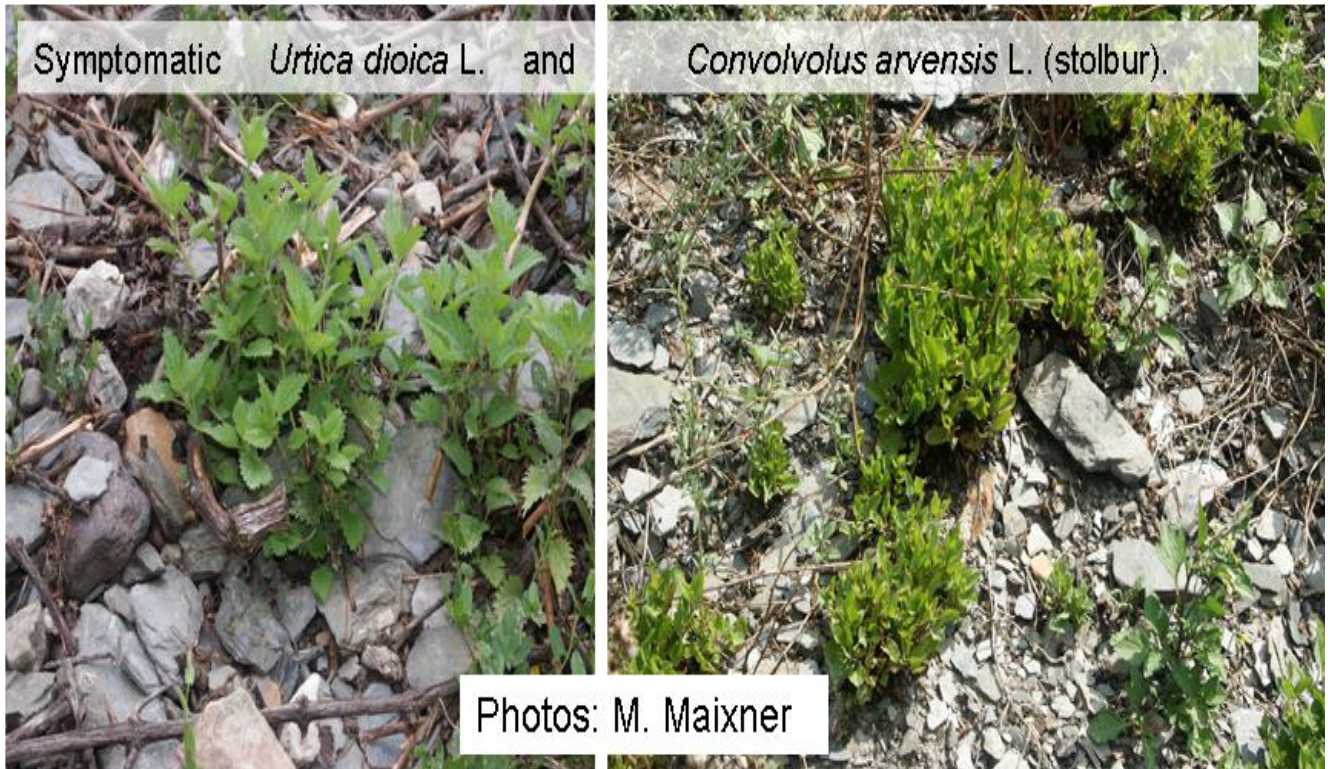
hosts, but *Convolvulus*, *Cirsium* and probably others may show yellowing, stunting and other phytoplasmoses-like symptoms. Preferential sampling of symptomatic ones may even reveal new hosts with epidemiological importance.

When collecting weeds, make sure that they are properly identified. For example, *Calystegia sepium* has been, to our experience, mistakenly identified in preflowering phase as *Convolvulus arvensis*. Identification of *Convolvulus althaeoides* (below, right) in the Mediterranean should be easier in case this bindweed type plant is found symptomatic and/or phytoplasma infected.

Uninfected *Convolvulus arvensis* L. and *Calystegia sepium* L.



CroFlora database



#### 4. 2. Tissue storage and DNA extraction

Practical considerations of weed sampling and phytoplasma extractions include preferential methods of tissue storing and DNA extraction. Storing the newly collected herbaceous material in a cool box prior to extraction or freezing (-20°C for short term, or -80°C for longer) should keep the level of tissue oxidation at minimum. We had good experience with drying bigger veins (where applicable) or complete leaves on calcium-carbonate in the field. DNA extraction, as pointed out in Langer and Maixner (2004) for nettle, may be difficult. The level of polyphenols and polysaccharides in weeds is often high and it should be considered when the experiments are planned.

#### 4. 3. Collection of other herbaceous plants

Solanaceous crops, celery, strawberry and lavender are not represented (except for 1 potato sample) in the current version of the database. Efforts should be made to collect some symptomatic plants especially if located in the areas of grapevine and/or vegetable and aromatic plants cultivation.





Stolbur symptoms on potato (left) and tomato (right).

Lavender and aromatic plants (thyme – found to be a stolbur host in Spain, chamomile, *Chrysanthemum cinerariifolium*) have become new cash crops in continental Croatia (Slavonia) lately. It may be, or soon became, the case in other countries of this network. Their cultivation plots often border with vineyards (lavender) or vegetables, maize, and sometimes even tobacco, all known stolbur hosts. Plants with little leaves, yellows, virescence, stunting and general decline should be seriously considered for sampling. Solanaceous crops with exemplary stolbur symptoms like the tomato and potato shown above should not be missed.

#### 4.4. Conclusion

Underrepresented plant isolates of stolbur in the database have been pointed out in the text above. Ideally, the whole pathosystems should be considered which translates to a simple rule - collecting weeds, cultivated plants and insects in the same area whenever possible. Accordingly, recommendations given in the **Subtask 3.2.** (Collection of Cixiids) for sampling sites should be considered.

#### Acknowledgements

I am grateful to Dr. Michael Maixner for his help with this subtask and photographic documentation. Dr. Antun Alegro is acknowledged for his botanical expertise and inspiring stories on plants.

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Collective sources of information:

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Also available at ICVG site <http://www.icvg.ch/>

First International Phytoplasmaologists Working Group Meeting 2007 Atti di 3. incontro nazionale sulle malattie da fitoplasmi (2005), Università degli studi di Milano, Istituto di patologia vegetale, Italia.

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CroFlora database <http://www.hirc.botanic.hr/croflora>



# **Global epidemiology of phytoplasma diseases of economic importance in Southeast Europe**

## **Task 3. Typing of stolbur phytoplasma isolates (group 16SrXII-A)**

### **SubTask 3.2 Collection of cixiids**

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## 1. Background: Cixiids (Vectors)

Cixiidae is a species-rich family with worldwide distribution. Over 100 species exist in Europe, out of which about one third occurs in central Europe. Adults of European species are usually polyphagous, feeding on trees and shrubs (Holzinger *et al.* 2003). Females oviposit into the soil, nymphs are with endogeic development and feed on roots.

Cixiid species known as or possible vectors of Stolbur phytoplasma are: *Cixius wagneri* (China) (Fig.1), *Hyalesthes obsoletus* Signoret (Fig.2), *Pentastiridius beieri* Wagner, *P. leporinus* (Linnaeus) and *Reptalus panzeri* (Löw) (Fig 3, above and left).



Fig. 1. *Cixius wagneri* (China)



Fig. 2. *Hyalesthes obsoletus* Signoret

Several *Reptalus* species had been identified as relatively common and probably may have role in phytoplasma transmission: *R. quinquecostatus*, *R. cuspidatus*, *R. melanochaeta* (Fig. 3; Appendix –Table 1). Presence of Stolbur in *R. quinquecostatus* is already documented (Trivellone *et al.*, 2005).



*Reptalus panzeri*



*Reptalus cuspidatus*



*Reptalus melanochaeta*



*Reptalus quinquecostatus*

Fig 3. *Reptalus* species - potential vectors of Stolbur

## 2. Sampling

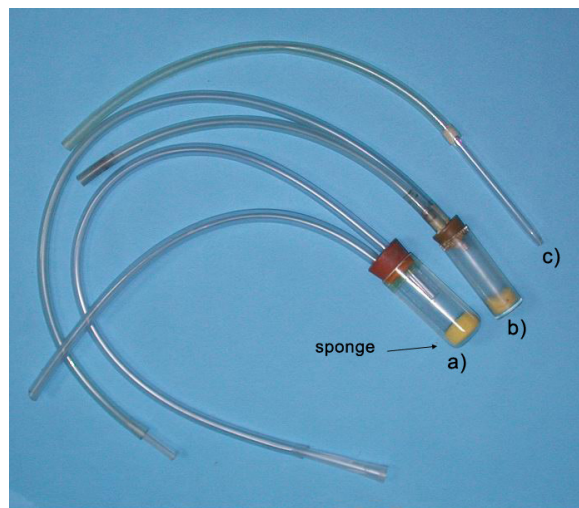
Real estimation of population density of Cixiids on selected sites is correlated with ability to collect their representatives. Standard methods, such as exposing yellow sticky traps, do not provide significant information about cicadas abundance. According to our experience, swiping during daylight is probably the best method which can give information on Cixiids presence, as well as, possible association with plants that can serve as hosts to adults. Cixiids are more active during night, so their evident phototrophic behavior usually gives quite valuable information about number of species inhabiting site and their abundance. For this reason, we propose as efficient methods both, daylight and nightlight sampling of Cixiids.

### 2.1. Daylight sampling method

**Collecting equipment:** robust sweeping net with solid frame (40-50cm diameter, 50 cm depth, Fig 4), mouth exhauster (Fig. 5), vials with 80% ethanol, cool-boxes. When collecting living material for laboratory study, captured samples should be placed in previously prepared plastic cylinders with mixture of several most common plants (potted), found at collecting site.



Fig 4. Sweeping net



- a) field sampling - "big" cixiids
- b) field sampling - "small" cixiids
- c) laboratory handling

sponge- for catching live and undamaged cixiids

Fig 5. Mouth exhauster

**Collecting strategy 1:** "15 minutes walk" in zigzag transect (intervals 20m) may give general view of Cixiids abundance on selected site. Collected material may give data on the general abundance of the cicadas on selected site. It is strongly recommended that material during "15 minutes walk" should be separately marked for statistic calculations.



**Collecting strategy 2:** consider sweeping on specific phytocenological assembly, where collector selects spots inside the site where Cixiids are more abundant. This is important moment during collection, because it provides information on possible plant-vector interactions.

## 2.2. Nightlight sampling method

**Collecting equipment:** electric power mobile unit, light source (metahalogen), white cloth, exhauster (Fig. 6).



Fig. 6. Nightlight sampling equipment

**Collecting strategy:** consider collecting Cixiids which are attracted to light. Usually, nightlight collections are more productive and identify more Cixiid species which are not collected by daylight sweeping (hidden behavior of some species), or bring data concerning abundance and number of species inhabiting collecting site. Increasing activity of Cixiids (in general) starts with dusk, while activity of Cixiids during night may perform different flying periods (flying on light in “waves”).

## 2.3. Larvae sampling method

Consider collection of larvae on sites where population of single Cixiid species is expected or targeted collection of larvae on selected plants (roots) to study vector-plant interaction. Cixiids larvae are situated 15-30 cm deep in the soil, usually gregarious (2-5 larvae) feeding on terminal roots of the host. As a good indicator of its presence are remains of white wax along feeding spots on roots (Fig.7 A, B). Prior to emergence, L5

### 3. Identification

Most recent publication that deals with taxonomy of European cixiids is:

Holzinger, W. E., Kammerlander, I., & Nickel, H. (2003). *The Auchenorrhyncha of Central Europe, Fulgoromorpha, Cicadomorpha Excl. Cicadellidae* (p. 673). Leiden: Brill Academic Publishers.

### 4. Sampling sites

- Sampling should be done on at least two target sites (agro-ecosystems).

a) Every target site should consider site which is affected with phytoplasma (endangered agro-ecosystem) and a nearby sites (s.l.) which may be significant from epidemiological point of view.

b) Agro-ecosystems of significance are: vineyards, corn fields, strawberry fields, pepper, potato and tomato crops.

c) It is recommended that selected nearby sites should have physical connection to target site (forest margin, meadow along the crop, etc).

d) It is recommended that sampling should be done in 15 days intervals (from beginning of April to end of September) to provide data of Cixiids fluctuation during collecting season.

d) Forest margins should be also considered as sampling sites as natural habitats of most little-known but common Cixiids.

## 5. Sampling protocol

Locality :			
GPS:			
Locality code:			
Date:			
Time:	Day sampling time: hh-mm	Night sampling time: hh-mm	
Site description:  Site exposition:			
Site phytocenosis/assembly:			
Dominant plants (site cover)	>30%	10-30%	<10%
Number of cixiids species observed:			
Observed cixiids-plant associations	Cixiid sp.	Plant sp.	
	.....	.....	
	.....	.....	
	.....	.....	
15 minutes sampling	code of vial:		
Remarks:			





#### **Task 4 : Typing Typing of fruit tree phytoplasma isolates (group 16SrX)**

**Coordinator :** Wolfgang Jarausch, RLP-Alplanta, Germany

**The context :** European stone fruit, pear decline and apple proliferation phytoplasmas (group 16SrX) are affecting South European orchards and are transmitted by psyllids, some of which are doing part of their ecological cycle on wild hosts. Genotyping phytoplasma isolates at the strain level will help to trace the route and the origin of phytoplasma diseases outbreaks.

**Objectives :** Establish a list of fruit tree phytoplasma DNA isolates collected on plant hosts or insect vectors. Evaluate the genetic diversity of group 16SrX phytoplasmas at the European geographical scale.

**Methodology :** DNA extracts from the established list will be submitted to phytoplasma-specific PCR detection test to verify their infection status. Genotyping of the isolates by using the four genotyping tools based on sequencing of *aceF*, *pnp*, *secY* and *imp* genes .

**Task Input :** DNA extracts from infected fruit trees and psyllid vectors.

**Result, milestones :** 1. Final first isolate list and markers, january 2008. 2. Final list of isolates including psyllids, May 2008. 3. Molecular typing of phytoplasma isolates. July 2008.

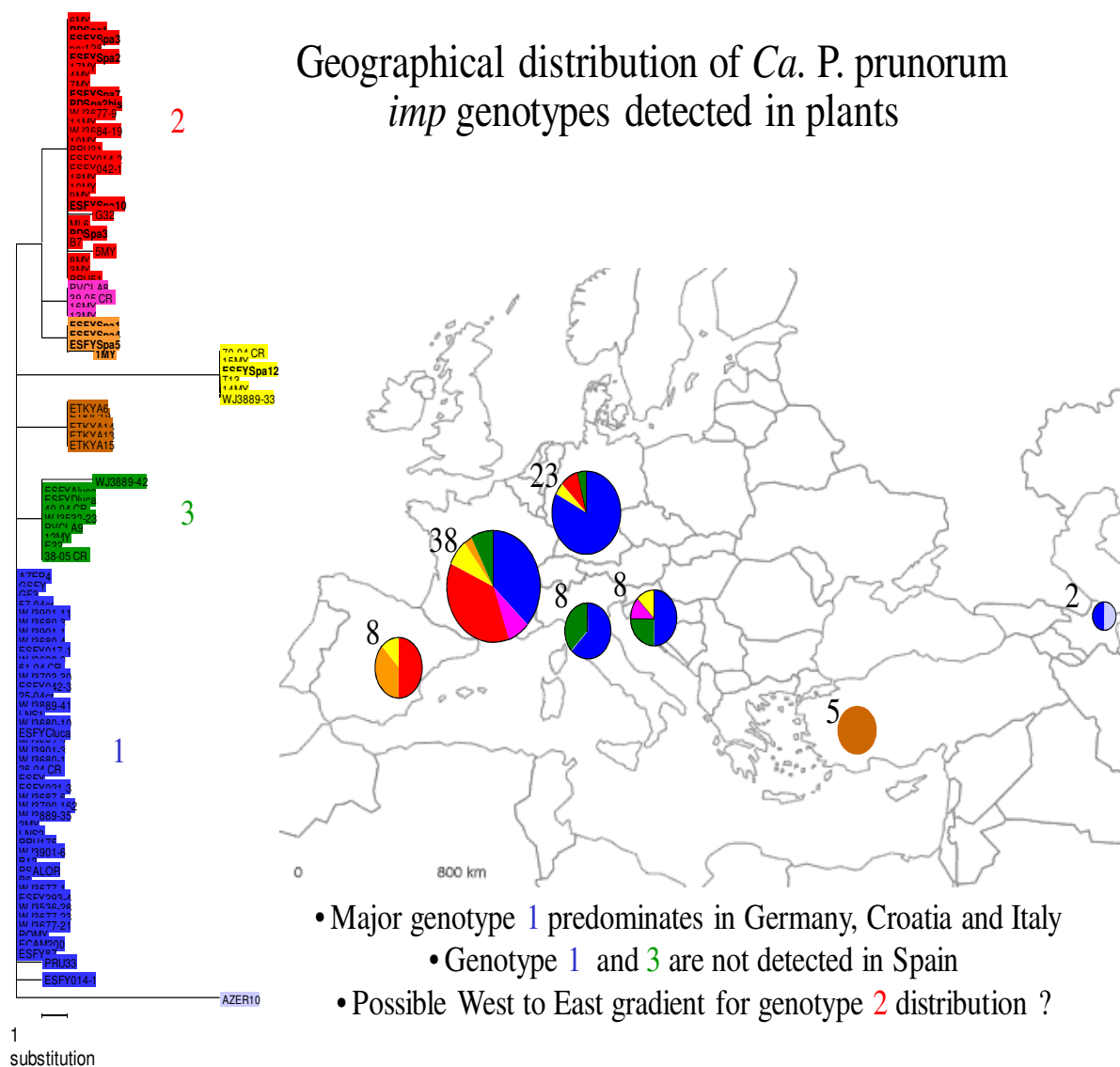
##### **1. Constitution of the list of samples**

A set 74 of *Prunus* samples infected with *Ca P. prunorum* (ESFY), 25 *Malus* samples infected by *Ca. P. mali* (AP) and 35 *Pyrus* samples infected with *Ca. P. pyri* (PD) were collected in Germany, France, Serbia, Hungary, Greece and Croatia. This list was completed by a set of isolates coming from Spain, Italy, Azerbaijan and Turkey.

Psyllids DNA were also extracted from 64 insects positive for ESFY (42), AP (25) and PD (3).

## 2. Plant and insect isolates genotyping :

Most of the samples could be amplified for the four gene markers and sequenced. Each type sequence was given a genotype number according to the following nomenclature. First letter of the marker and the number of the genotype. For example : A1 is the first genotype for the *aceF* marker. The most of the diversity was found for the marker *imp* for which 28 different genotypes were found. An example of the analyses which are going to be performed is shown in the figure below.





## Task 5 : Development of mitochondrial markers for insect typing

**Coordinator** : Nicolas Sauvion, belonging to team INRA-Montpellier-France

### The context :

The quarantine grapevine phytoplasma inducing Flavescence dorée (FD, group16SrV) and transmitted by a leafhopper of North American origin, has been recently reported in Balkans and the recent genetic characterization of the related alder phytoplasmas indicate they could constitute a wild reservoir of FD. The stolbur phytoplasma (group 16SrXII-A) is a phytoplasma endemic to Europe and Near East and originate from the wild compartment (bindweed, nettle, ...) from which it is transmitted to grapevine, maize, solanaceous crop (tomato, pepper, potato, eggplant), strawberry, lavender and sugarbeet by polyphagous planthoppers of the Cixiidae family. Some stolbur phytoplasma strains were recently found to be associated with specific insect vector ecotypes, which could indicate some strains specialization. Finally European stone fruit, pear decline and apple proliferation phytoplasmas (group 16SrX) are affecting South European orchards and are transmitted by psyllids, some of which are doing part of their ecological cycle on wild hosts.

Only specialized entomologists can differentiate these vectors of phytoplasma; in particular the psyllids because of the morphological similarity of the *Cacopsylla* species. It is only recently than a electronic key have been developed for an easy determination of these psyllids by plant protection services, skilled growers or researchers (EUREKA project 'Psyllids vectors' see : <http://www.psyllidkey.info/>). No key of this type exists for the other vectors. This limitation inherent in morphology-based identification systems signal the need for a new approach to the vectoring species. DNA-based identification systems are today reliable, cost-effective and accessible solution for species identification (e.g. Hebert 2003).

The most commonly sequenced regions in insect systematics are mitochondrial DNA (mtDNA) and nuclear ribosomal DNA (rDNA). As contiguous pieces of DNA, these two classes also lend themselves to easy comparison (Caterino et al 2000). The combination of nuclear and mitochondrial data is useful because incongruence between nuclear and mitochondrial phylogenies can reveal important aspects of species histories such as introgression, hybridization, direct or indirect selection, and incomplete lineage sorting (for reviews see Simon et al 2006). Because mtDNA can introgress faster than nuclear DNA and can sometimes entirely replace the mtDNA of the species invaded the combined use of nuclear traits is an important check.

Mitochondrial genes are useful tools for the phylogenetics of closely related taxa (Simon et al 2006), and so they seem to be appropriate to differentiate the vectors of phytoplasma. One of the most frequently sequenced mitochondrial gene is cytochrome oxidase I (COI). However, due to its length, the specific region chosen varies from study to study. Sequencing larger fragments (more than 1 kb) increases the likelihood that regions of different levels of variation will be spanned, giving resolution for a greater range of divergences. But, for a question of time and cost, in a first time we have decided to choice a specific region of 708 bp classically used for Hemiptera.

The picture for nuclear rDNA is one of greater consistency (Caterino et al 2000). The internal transcribed spacer (ITS) and 28S regions predominate in studies of Diptera and Hymenoptera, and satisfactory results have been recently obtained with Hemiptera like aphids or whiteflies. ITS has typically been most useful for molecular systematics at the species level, and even within species (e.g., to identify geographic biotypes).

**Objectives** : At the beginning of the project, it was decided to focus not only on the development of a mitochondrial marker (COI) but also on a nuclear marker (ITS) for molecular typing of the vectoring species.

**Methodology** : DNA extracts from the established list will tested during the workshop with two pair of primers :

(1) COI primers : LCO-1490 / HCO-2198 (Folmer et al.1994) ; (2) ITS primers : FCM / BrD (Hillis & Dixon 1991). At the beginning of the project (first meeting Bologna), the choice have been done to test a range of samples of each species from different localities (that is, most large number) rather than many individuals of few localities. Primers (COI and ITS) have been choice after preliminary successfully tests on *Cacopsylla pruni* in the laboratory of Montpellier.

**Task Input :** DNA extracts from *Scaphoideus titanus*, *Hyalesthes obsoletus*, *Reptalus panzerii*, *Cacopsylla pyri*, *C. pruni*, *C. melanoneura* and *C. picta*.

**Result, milestones :** 1. Molecular markers identified. April 2008. 2. Molecular typing of vectoring species sampling in France, Germany, other countries if applicable.

## 1. Constitution of the list of samples

In December 2008, the following call was sent to all the participants in order to constitute the list of DNA samples :

- *Cacopsylla* spp.: countries where European Stone Fruits Yellow, Apple Proliferation and Pear Decline are recorded should collected insect samples representative of the main focal points on their territory. Five to 10 individuals per site, and ideally two to 3 samples by country should be sufficient.

- *Scaphoideus* spp.: countries having Flavescence dorée should collected insect samples representative of the main focal points on their territory. Five to 10 individuals per site, and ideally two to 3 samples by country should be sufficient.

- *Hyalesthes* spp.: countries having Bois Noir should collected insect samples representative of the main focal points on their territory. Five to 10 individuals per site, and ideally two to 3 samples by country should be sufficient.

During the first meeting in Bologna, 6 teams of the project confirmed their participation in the task 2 : INRA-Montpellier, France, INRA-Bordeaux, France, RLP-AIPlanta, Germany, BBA Bernkastel, Germany, DBUF-IPPAF, Croatia, IPPE-Zemun, Serbia, and a associated partenaire, Palacky University of Olomouc, Czech Republic.

INRA-Montpellier and RLP-AIPlanta transmitted a first list of *Cacopsylla* (*C. pruni* and *C. picta*, but not only) collected in different sites in France and Germany isolates from *Prunus* sp. or conifers, and from which the DNA was already extracted.

Palacky University of Olomouc declared his intention to send to the team of Montpellier samples in alcohol of whole *C. pruni* collected in different sites in Czech Republic on *Prunus* sp. or conifers.

INRA-Montpellier declared his intention to test several primers (COI and ITS) with DNA extracts of psyllids or cixiids.

At Bordeaux, in June during the Workshop, the final list of samples was established according to the format collectively established during the Bologna meeting and was distributed to all the participants. The list in the annex is a summary/synthetic list.

A total of 214 individuals were analysed. Few *Cacopsylla* spp. from France were tested during the Workshop. In fact, only some of them were used as positive control. INRA Montpellier have a collection of thousand samples, and sequencing are in progress within the framework of another project (Barcoding project). Details concerning sampling sites, number of individuals per site etc are given in the annex. We were able to collect psyllid samples only of 3 countries : Croatia, Czech Republic and Germany. This denotes actually the difficulty to find entomologists capable of making the collects. We can do the same remark for the other vectors : *Scaphoideus titanus* were collected by Daciana Pupara (INRA-Bordeaux), and *Hyalesthes titanus* by Michael Maixner (BBA Bernkastel) or Xavier Foissac (INRA-Bordeaux)

## 2. DNA extractions

DNA was isolated individually using a cetyl-trimethyl ammonium bromide (CTAB) protocol (Doyle & Doyle 1987) for almost all insects analysed in this project. DNA of several psyllids (see annex) were extracted with the methodology of prepGEM™ Insect (for details see <http://www.zygem.com/Products/Products-PG-Insect.html>) at Montpellier or during the Workshop.

## III. Typing of the insects

The majority of the *C. melanoneura* gave positive results with ITS primers, but negative results with COI primers, independently of the origin of psyllids.

*C. picta* gave good results with ITS primers, and COI primers, except for insects from Czech Republic with COI.

Disappointingly, typing of *C. pruni* gave negative results (except for positive control!) with ITS and COI, except for individuals from Germany with COI. We cannot exclude a problem because of the DNA extraction with the Zygem protocol for the Czech samples. New extractions from these localities will be done with the classical (CTAB) protocol to test again the ITS and COI primers.

The other psyllid species (*C. pyri*, *C. pyricola*, and *C. pyrisuga*) gave negative results for almost all samples. Bad DNA extraction or conservation of the extracts could explain these results (almost all samples were coming from the same lab) but we cannot exclude a problem with the primers. These were non-degenerated and because of their specificity, they may fail if there are mismatches in critical positions. It should be useful to test degenerated primers.

ITS and COI primers used in this study seem a good tools for *Scaphoideus titanus*. Except for several individuals, all samples gave positive results.

On the contrary, almost all the *Hyalesthes obsoletus* samples are negatives, particularly those which were extracted with the Zygem protocol. New extractions should be done with the classical (CTAB) protocol to test again the ITS and COI primers before any definitive conclusion concerning the interest of the used primers.

### 3. Conclusion and perspectives

Molecular typing with psyllid species realised at the Montpellier laboratory showed that the results with COI or ITS were very variable from a species to the other one, even between individuals of the same species. Results described here confirm this observation. This shows the difficulty finding the adequate primers. To by-pass this problem two strategy could be followed : (1) use degenerated primers at one or more sites (see Simon et al 2006 for a large list of primers) ; (2) refine primers by sequencing the region surrounding the primer for representatives of the desired taxa (by using bracketing primer pairs), and reducing or eliminating degeneracy where possible. These two strategies will be tempted at INRA-Montpellier, at least in a first step on the psyllids.

We cannot exclude that the negative results could also due to a bad quality of the DNA (because of the extraction or mode of conservation). Participants of the Workshop had brought whole insects in the alcohol, so extractions will be done again with the classical (CTAB) protocol at the INRA-Montpellier lab.

Independently of the technical problems met during this study, a major conclusion is, it was very difficult to collect insects from the various partner countries of the project. This prove a real and crucial problem in finding people for trapping insect in the field.

All the samples from which we have had positive results, will be sequenced within the framework of the Barcoding project ([http:// www.barcodinglife.org](http://www.barcodinglife.org)) in collaboration with J-Y. Rasplus (INRA-Montpellier, CBGP, <http://www1.montpellier.inra.fr/CBGP/rasplus.html>).

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- Hillis DM, Dixon MT (1991) Ribosomal DNA — molecular evolution and phylogenetic inference. Quarterly Review of Biology, 66, 410-453
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Species	Country	Sampling site	N	ITS	COI	Sampler	Extraction
<b>Vectors of Apple Proliferation</b>							
Cacopsylla melanoneura	Croatia	Jastrebarsko	5	3+	4-	DS	zygem
Cacopsylla melanoneura	Croatia	Dolci	1	-	nd	DS	ctab
Cacopsylla melanoneura	Czech Republic	Olsany	5	3+	5-	RF	zygem
Cacopsylla melanoneura	Czech Republic	Perna	5	3+	5-	RF	zygem
Cacopsylla melanoneura	Czech Republic	Valesovici	5	2+	5-	RF	zygem
Cacopsylla melanoneura	Czech Republic	Brno	5	5-	5-	RF	zygem
Cacopsylla picta	Germany	Obernai (Alsace) "Ver2"	2	2+	1+	BJ	ctab
Cacopsylla picta	Germany	Magden (Suisse) "CH1"	1	+	+	BJ	ctab
Cacopsylla picta	Germany	Karlsruhe (D) "KA1"	2	2+	2+	BJ	ctab
Cacopsylla picta	Germany	Offenburg (D) "OG2"	1	+	-	BJ	ctab
Cacopsylla picta	Germany	Neustadt "NW1"	10	7-	7+	BJ	ctab
Cacopsylla picta	Germany	Südpfalze (D) "Inter2"	2	2+	2+	BJ	ctab
Cacopsylla picta	Germany	Freiburg "FR8"	2	2-	1+	BJ	ctab
Cacopsylla picta	Croatia	Jastrebarsko	5	2+	1+	DS	zygem
Cacopsylla picta	Czech Republic	Olsany	5	3+	5-	RF	zygem
<b>Vectors of European Stones Fruits Yellow</b>							
Cacopsylla pruni	Germany	Koblenz "Gräb8"	1	+	-	BJ	ctab
Cacopsylla pruni	Germany	Neustadt "U2"	5	5-	5+	BJ	ctab
Cacopsylla pruni	Germany	Oppenheim "OP2"	1	-	+	BJ	ctab
Cacopsylla pruni	Germany	Koblenz "Gräb84"	2	2-	2+	BJ	ctab
Cacopsylla pruni	Czech Republic	Velesorice	16	3+	1+	RF / NS	zygem
Cacopsylla pruni	Czech Republic	Brno, Cerveny Kopee	8	3+	3+	RF / NS	zygem
Cacopsylla pruni	Czech Republic	Bulhary	8	1+	2+	RF / NS	zygem
Cacopsylla pruni	Czech Republic	Drahanska vrchovina, Kaleeni	8	1+	3+	RF / NS	zygem
Cacopsylla pruni	France	Fesche	2	2+	2+	NS	ctab
Cacopsylla pruni	France	Larzac	2	2+	2+	NS	ctab
Cacopsylla pruni	Croatia	Skudelin	1	-	-	DS	ctab
<b>Vectors of Pear Decline</b>							
Cacopsylla pyri	Croatia	Osijek	9	1+	1+	DS	zygem
Cacopsylla pyri	Croatia	Jastrebarsko	1	+	+	DS	ctab
Cacopsylla pyri	Croatia	Skudelin	1	-	+	DS	ctab
Cacopsylla pyri	Croatia	Sv. Marija	1	-	+	DS	ctab
Cacopsylla pyri	Croatia	Jastrebarsko	1	-	+	DS	ctab
Cacopsylla pyri	Croatia	Vucjak Fer.	1	-	-	DS	ctab
Cacopsylla pyri	Croatia	Cice	1	-	-	DS	ctab
Cacopsylla pyricola	Croatia	Jastrebarsko	2	2-	2-	DS	ctab
Cacopsylla pyrisuga	Croatia	Jastrebarsko	5	2+	5-	DS	zygem
Cacopsylla pyrisuga	Czech Republic	Olsany	13	6+	1+	RF	zygem
Cacopsylla pyrisuga	Croatia	Voloder	1	-	-	DS	ctab
Cacopsylla pyrisuga	Croatia	Osijek	1	-	-	DS	ctab
Cacopsylla pyrisuga	Croatia	Vucjak Fer.	1	-	-	DS	ctab
<b>Vectors of Flavescence dorée</b>							
Scaphoideus titanus	Rumania	Timisoara	2	1+	2+	DP	ctab
Scaphoideus titanus	Canada	Saskatoon	2	2-	2+	DP	ctab
Scaphoideus titanus	USA	Hither Hills	5	5+	5+	DP	ctab
Scaphoideus titanus	USA	Niagara Peninsula	11	9+	10+	DP	ctab
Scaphoideus titanus	Rumania	Iasi	2	2+	2+	DP	ctab
Scaphoideus titanus	Croatia	Ilok	2	2+	1+	DS	ctab
Scaphoideus titanus	Croatia	Strigova	1	-	-	DS	ctab
Scaphoideus titanus	Hungary	Kiskun halas st.	2	2-	2-	IE	zygem
<b>Vectors of Bois noir</b>							
Hyalesthes obsoletus	Germany	Bernkastel-Kues	40	14+	24+	MM	zygem
Hyalesthes obsoletus	France?	France?	35	2+	1+	XF	zygem
Hyalesthes obsoletus	Croatia	Zelezna gora	1	-	+	DS	ctab
Hyalesthes obsoletus	Croatia	Voloder	2	-	-	DS	ctab
Hyalesthes obsoletus	Croatia	Drnis	1	-	+	DS	ctab
<b>Vectors ?</b>							
Asymetrasca decedens	France	Verteuille	1	-	+	DP	ctab
Empoasca decipiens	France	Verteuille	2	1+	2+	DP	ctab
Empoasca solani	France	Verteuille	2	2+	1+	DP	ctab
Empoasca vitis	France	Verteuille	2	2+	2+	DP	ctab
Jacobiasca libica	Portugal	?	2	2-	2+	DP	ctab

DS : Ivana Krizana / Dijana Skovic (HR)  
 BJ : Barbara Jarausch (D)  
 RF : Renata Fialova (CZ)  
 NS : Nicolas Sauvion (FR)  
 DP : Daciana Papura (FR)  
 XF : Xavier Foissac (FR)  
 MM : Michael Maixner (D)  
 IE : Ibolya Ember (HU)

all negative  
 almost all negative  
 50% positive  
 almost all positive  
 all positive

N number of tested individuals



## Global epidemiology of phytoplasma diseases of economic importance in Southeast Europe (Network PHYTOPLASMA-EPIDEMIO)

### **Tasks 6. Website and database for phytoplasma strains description**

**Task Leader : Florin Oancea (team ICDPP Bucharest Romania)**

The network “*Global epidemiology of phytoplasma diseases of economic importance in Southeast Europe*” coordinate the efforts of plant pathologists, microbiologists and entomologists of Southeast European countries to better monitor phytoplasma strains propagation through nurseries and insect vectors, at the European scale. The activity 6 was to develop a website of the project, which is hosting also the database for phytoplasma strains description.

The web site of the project is intended to reach two aims:

- ✓ Communicate to the stakeholders of the importance of the ERANET project regarding the global epidemiology of phytoplasma diseases of economic importance in Southeast Europe”;
- ✓ Exchange results between scientists members of the network.

The following major objectives were considered to be delivered by the web site:

- ✓ The website had to be dynamic, by incorporating a database, to allow the simple and secure communication of results between Phytoplasma epidemio project members.
- ✓ The website had to be easily updatable by the project leader / activity leaders until completion of the scientific project.
- ✓ The website had to be informative, by providing a section which would provide relevant information about project.
- ✓ The website had to be accessible, also considering the needs of those with low bandwidth connections and older technologies so that they can use, interact, understand, navigate and fully perceive the website.

The main characteristics of the web site of the projects were considered to be: (i) fast-loading; (ii) flexible; (iii) functional and (iv) accessible. A special attention was paid to the way of presenting the information. This was effectively presented in a front-loading manner, called the inverted-pyramid. This style ensures paragraphs are kept short, presenting one main point at a time. Each paragraph begins with the conclusion, and then presents supporting details.

The web site includes two parts: the public one, including the information about the Phytoplasma epidemio network and project, participants, objectives, news. The private part of the network is accessible only by username and password, for the members of the SEE ERANET network. This part include a database developed as a MySQL object under a PHP environment running on an Apache server.

The developed public part of the website comply with the W3C accessibility guidelines which provide equal access and equal opportunity for disadvantaged or disabled users (Caldwell *et al.*, 2008). The W3C is the governing body for developing protocols and guidelines for the web, including the standards that drive Hypertext Markup Languages and stylesheets. The W3C accessibility guidelines mean that more websites and software are available for people with disabilities to use the web more effectively. (Caldwell *et al.* 2008). On the public part the information is structured in 9 pages: Home, Project, Objectives, Results, Activities, Team, Duration, Participants, Events.

The web page is presented in fig.1. The design of the web page was chosen by Phytoplasma epidemio project members from 29 different website designs.

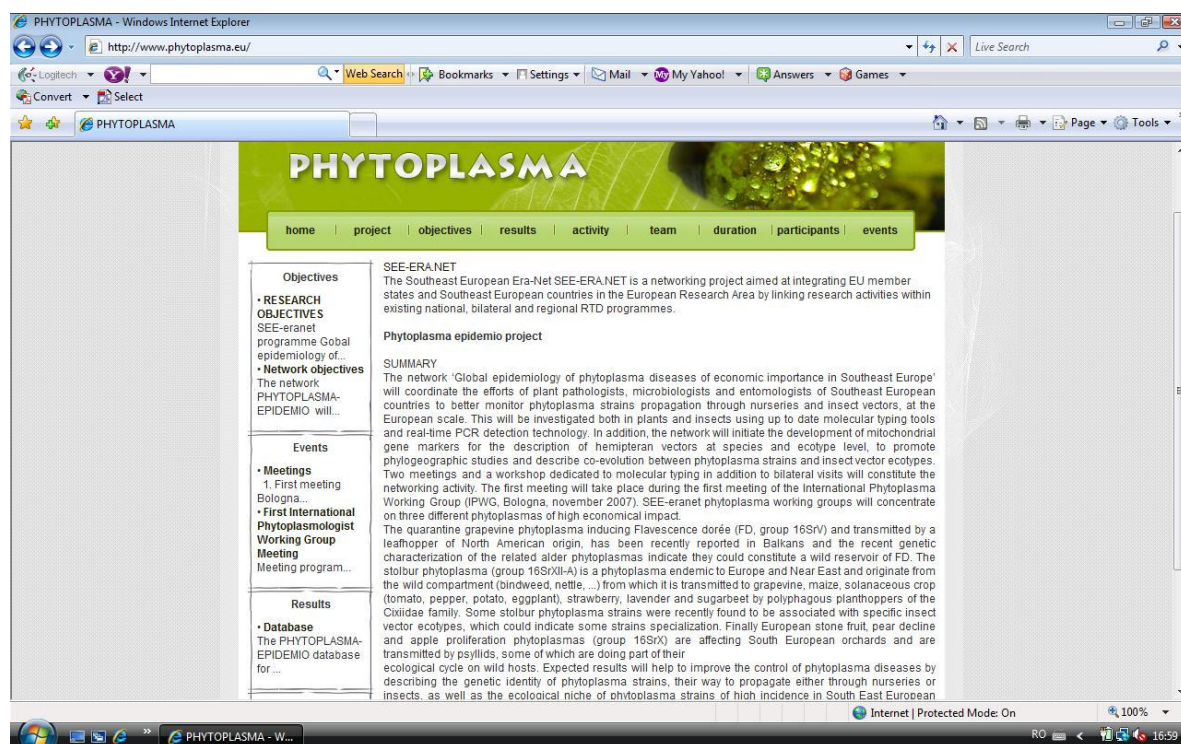


Fig.1. Website of the “*Global epidemiology of phytoplasma diseases of economic importance in Southeast Europe*” – network Phytoplasma epidemio.

On the Home page it is a header with link to the 9 pages of the web site. This page also include a summary description of the Phytoplasma epidemio project; a link to objectives; a link to events pages; a link to results pages (which include the link to private section of the web site - database).

On Team page it is a list of research entities participating to the Phytoplasma epidemio project, with link to the webpage of each entity and on Participant page is a list of the research entities with secured link to the participant e-mail address.

On page Results are short presentations of the reports on each activity, with link to the complete forms of reports encapsulated as (locked) pdf. Here is also the entrance to the database (secured link to the database, based on password). On Activities page there are presentation of



project activities objectives and task leaders (with secured e-mail link, opening an Outlook form).

In order to create the database of the project Phytoplasma epidemia first of all was chosen the DBMS (Database Management System), i.e. the software that defines a database, stores the data, supports a query language, produces reports, and create data entry screens. The EU public funding of this network requires the use of open-source software applications PHP, MySQL, and Apache. PHP: HyperText Preprocessor, stands for Personal Home Page tools and is a scripting language designed to be embedded in HTML code (Valade 2002). When a user requests to see a HTML static website, the web server sends the HTML code as-is which is then read by the web browser. In contrast, code containing PHP on a dynamic site is processed by PHP software such as Apache, before it is returned to the web browser. It is this processing which allows for complicated tasks such as data entry on a dynamic site. The processed data can be stored with MySQL, a small, efficient, easy-to-use and secure open source database popular with Web developers. A database acts as a long-term memory that stores information for an application, similar to an electronic filing cabinet.

Naramore *et al.* (2005) made a creative analogy for an explanation of how Apache, MySQL and PHP work together in a dynamic website. They represent the website as a fancy restaurant. The customers, or users of the site, want their food to appear attractive, but do not care how the food is prepared. The customer does not want a buffet-style meal where they make their own meal (or develop the website themselves). This restaurant encourages an interaction between the customers and waiter, thereby ensuring complete customization of food according to dietary needs. In this analogy, the waiter, PHP, goes to the kitchen with specific instructions for the food preparation. The chef in the kitchen is Apache, preparing different types of food (or files) in a quick and flexible manner. MySQL is the stockroom containing all the ingredients (or the database of information).

The phytoplasma isolate properties to be described for the insect isolate are the following: Nr. crt. (# ); Code; Disease; Running nb; Number (final); Isolate code; Extraction nb; Tube nb; Extraction date; Nb of insects; Country code; Department code; Growing region; Sampling site; Orchard name or code; GPS coordinates; Insect species; Developmental stage; Male; Female; Plant species; Direct PCR; Nested PCR; Universal primers; Specific primers; Name of specific primers; Final PCR result; Subtype rpS group; Marker1 xxx; Marker2 xxx; Marker3 xxx; Unambiguous association.

The phytoplasma isolate properties to be described for the plant isolate are the following: Nr. crt. (#); Code; Disease; Running nb; Nb; Isolate code; Extraction nb; Tube nb; Extraction date; Plant organ; Tissue; Country code; Department code; Growing region; Sampling site; Orchard name or code; GPS coordinates; Plant species; Plant age; Direct PCR; Nested PCR; Universal primers; Specific primers; Name of specific primers; Final PCR result; Subtype; rpS group; Marker1 xxx; Marker2 xxx; Marker3 xxx; Vineyard name or code; Localized or systemic symptoms; Weeds type of symptoms; Cultivar; No symptoms; Nonspecific symptoms; Specific symptoms; Global yellowing; Yellowing of some branches; Yellowing of the shoots; Small

The links for database are: <http://www.phytoplasma.eu/view/vizualizareins.php> (for insect phytoplasma database on SEE-ERA.NET Phytoplasma epidemio network) and <http://www.phytoplasma.eu/view/vizualizarepl.php> (for plant phytoplasma database).

[illegible]

### Form for entering insects data with FD-PGY

Running nb:  (integer with 6 digits, ex. 123456)

Nb:  (integer with 4 digits, ex. 1234)

Isolate code:  (string with maximum 30 characters)

Extraction\_nb:  (string with maximum 7 characters)

Tube nb:  (integer with 6 digits, ex. 123456)

Extraction date:  (data with format yyyy/mm/dd, ex. 2008/03/31)

Nb of insects:  (integer with 6 digits, ex. 123456)

Country code:  RO

Department code:  (string with maximum 4 characters)

Growing region:  (string with maximum 20 characters)

Sampling site:  (string with maximum 20 characters)

Orchard name or code:  (string with maximum 20 characters)

GPS coordinates:  (string with maximum 30 characters)

Insect species:  (string with maximum 30 characters)

Develop stage:  (string with maximum 10 characters)

Male:  (integer with 6 digits, ex. 123456)

Female:  (integer with 6 digits, ex. 123456)

Plant species:  (string with maximum 30 characters)

Direct PCR: ☐ (check it for TRUE value)

Nested PCR: ☐ (check it for TRUE value)

### Form for entering plants data with FD-PGY

Running nb:  (integer with 6 digits, ex. 123456)

Nb:  (integer with 4 digits, ex. 1234)

Isolate code:  (string with maximum 30 characters)

Extraction\_nb:  (string with maximum 7 characters)

Tube nb:  (integer with 6 digits, ex. 123456)

Extraction date:  (data with format yyyy/mm/dd, ex. 2008/03/31)

Plant organ:  (string with maximum 15 characters)

Tissue:  (string with maximum 15 characters)

Country code:  RO

Department code:  (string with maximum 4 characters)

Growing region:  (string with maximum 20 characters)

Sampling site:  (string with maximum 20 characters)

GPS coordinates:  (string with maximum 30 characters)

Plant species:  (string with maximum 30 characters)

Plant age:  (integer with 6 digits, ex. 123456)

Direct PCR: ☐ (check it for TRUE value)

Nested PCR: ☐ (check it for TRUE value)

Universal primers:  (string with maximum 10 characters)

Specific primers: ☐ (check it for TRUE value)

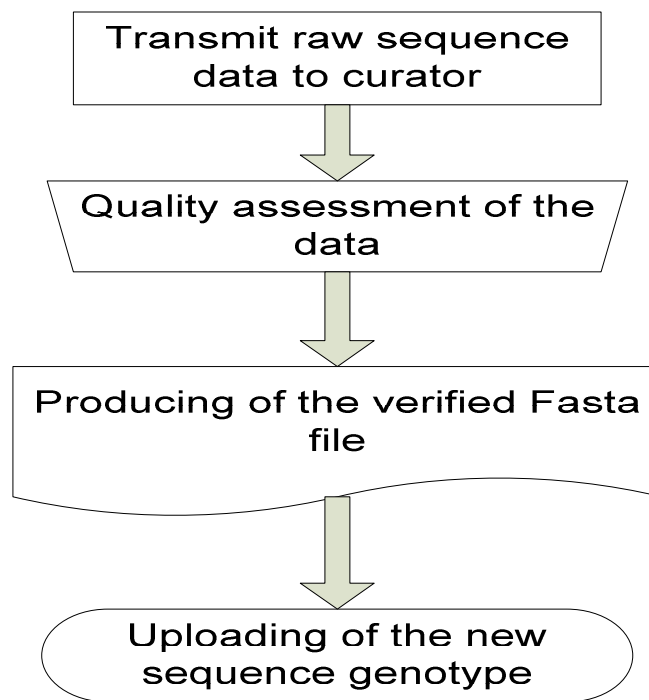
Name of specific primers:  (string with maximum 10 characters)

Final PCR result: ☐ (check it for TRUE value)

Subtype:  (string with maximum 10 characters)

**Fig. 2.** Aspect of pages of the web database and form for entering data for insect phytoplasma and plant phytoplasma. a) insects; b) plant.

In order to allow future development of the database a procedure for the description of a new sequence genotype was agreed with the members of the Phytoplasma epidemio network. This procedure is presented in fig.3.



**Fig. 3.** Procedure for the description of a new sequence genotype agreed with the members of the Phytoplasma epidemio network.

Among phloem-restricted bacteria, phytoplasmas are the most damaging plant pathogens with important economical impact in perennial as well as corn and legume crop. Phytoplasmas reside in sieve cells of plant phloem tissue and are transmitted by phloem-feeding hemipteran phloem feeders like leafhoppers, planthoppers and psyllids (Weintraub and Beanland, 2006), but also spread through nurseries when infected planting material is propagated. Very few genetic resistance to phytoplasma infection have been reported and disease control rely on prophylactic sanitary measures consisting in the destruction of infected crop, the chemical control of insects and protection of nurseries.

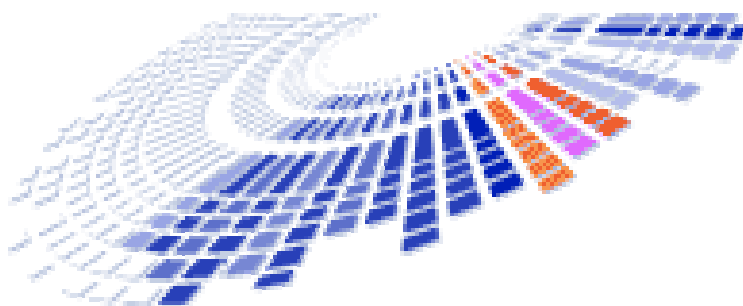
Genotyping of the phytoplasma European populations by using updated molecular typing tools is a solution for a better understanding and monitoring of these important economic plant diseases. Database are necessary for a proper analysis of the results from the molecular typing tools, and a dedicated website is an useful tool, both for communicating the importance of Phytoplasma epidemio network research and co-coordinating the activities of the participants to Phytoplasma epidemio network.

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## NETWORK

# GLOBAL EPIDEMIOLOGY OF PHYTOPLASMA DISEASES OF ECONOMIC IMPORTANCE IN SOUTHEAST EUROPE



## PRACTICAL WORKSHOP AND FINAL MEETING

**23-27 June 2008**

**UMR-1090 Génomique Diversité Pouvoir Pathogène  
INRA & Université Victor Ségalen Bordeaux2**



**IBVI**



# LIST OF PARTICIPANTS

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## PROGRAMME OF THE WORKSHOP

<b>Day1 (23-06)</b>		<b>In parallel</b>			
Virology meeting room	AM	9h30 Welcome addresses & Introduction 10h30 Phytoplasma collection – Visits of laboratory, greenhouses and insectarium			
	PM	Triplex Real time PCR Virology meeting room		Group 16SrX typing Jean-Luc's office	Insect Typing UMR Santé Végétale Bio-mol lab
<b>Day2 (24-06)</b>		<b>In parallel</b>			
	AM 9h	Triplex Real time PCR Xavier's Lab		Group 16SrX typing Jean-Luc's office	Insect typing UMR Santé Végétale Bio-mol lab
	PM 14h	FD and related typing Sylvie's Office	Stolbur typing Xavier's Lab & Mollicute meeting room	Group 16SrX typing Jean-Luc's office	Insect typing UMR Santé Végétale Bio-mol lab
<b>Day3 (25-06)</b>		<b>In parallel</b>			
	AM 9h	FD and related typing Sylvie's Office	Stolbur typing Xavier's Lab	Database and web site Virology Meeting room	Cixid and psyllids collection and/or taxonomy Jean-Luc's office
	PM 14h	FD and related typing Sylvie's Office	Stolbur typing Xavier's Lab & Mollicute meeting room	Database and web site Virology Meeting room	Cixid and psyllids collection and/or taxonomy Jean-Luc's office
<b>END OF PRACTICAL SESSIONS</b>					<b>DINNER IN BORDEAUX</b>
<b>Day4 (26-06)</b>					
Virology and mollicute meeting rooms	AM	Preparation of practical sessions reports Preparation of working group reports			
Virology meeting room	PM	Some scientific presentations on related TOPICS Reports for bilateral visits and practical sessions SEE-ERANET reports			
<b>Day5 (27-06)</b>					
Virology meeting room	AM	European perspectives : Formal propositions			
Virology meeting room	PM	European perspectives : Open discussion about perspectives & programmes Conclusions			

Lunches are at INRA restaurant between 12h and 13h30

## PRACTICAL SESSIONS PARTICIPANT LIST

Participants	PRACT I	PRACT II
<b>Sylvie Malembic-Maher*</b>	REALT-PCR	GRV
<b>Pascal Salar</b>	REALT-PCR	GRV
Dijana Skoric	REALT-PCR	GRV
Eleni Karatsiori	REALT-PCR	GRV
Katsiani Assimina	REALT-PCR	GRV
Emilija Nakova	REALT-PCR	GRXII
Varvara Maliogka	REALT-PCR	GRXII
Ibolya Ember	REALT-PCR	GRXII
Florin Oancea	REALT-PCR	DBWEB
<b>Xavier Foissac*</b>	PCR stolbur samples	DBWEB
<b>Anne Fabre*</b>	PCR stolbur samples	GRXII
Jes Johannesen		GRXII
Michael Maixner		GRXII
Renata Fialova	INSECT	GRXII
Barbara Jarausch	INSECT	CIX-PSYL
<b>Nicolas Sauvion*</b>	INSECT	CIX-PSYL
<b>Daciana Papura*</b>	INSECT	CIX-PSYL
Ivana Krizanac	INSECT	DBWEB
Wolfgang Jarausch	GRX	DBWEB
<b>Jean-Luc Danet*</b>	GRX	CIX-PSYL
Pavla Valova	GRX	GRXII

\*organizing committee

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### Wednesday 25 June Evening

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#### A Quick tour in Bordeaux Historical Center

**18h45** : meeting at the station 'Porte du Palais' on tram line A

#### Dinner in Bordeaux

**Restaurant 'La Petite Gironde' (cost 30 €)**

**meeting 20h** at square 'Stalingrad'

The address is 75 Quais de Queries. It is on the right river bank 10-15 minutes walk on left hand when crossing the bridge 'pont de pierre'.

Line A stop at 'Stalingrad' for the restaurant.

# **REPORTS & RELATED TOPICS**

## **Thursday 24 June morning**

### **Virology meeting room**

**9h00-10h30**      **Preparation of practical sessions reports :**  
**Produce a 5-10 min report**

Realtime-PCR : Virology meeting room	Ibolya Ember & Emilija Nakova
Typing of group V phytoplasmas : Sylvie's office	Dijana Skoric & Varvara Maliogka
Typing of group X phytoplasmas : Jean-Luc's office	Jean-Luc Danet & Pavla Valova
Typing of group XII phytoplasmas : Molli. meeting room	Anne Fabre & Jes Johannesen
Insect typing and taxonomy : Virology meeting room	Barbara Jarausch & Renata Fialova

**Preparation of working group reports**  
**Produce a 10 min report**

Typing of group V phytoplasmas : Sylvie's office	Sylvie Mal.-Maher & Dijana Skoric
Typing of group X phytoplasmas : Jean-Luc's office	Jean-Luc Danet & Wolfgang Jarausch
Typing of group XII phytoplasmas : Molli. meeting room	Michael Maixner & Slobodan Krnjajić
Insect typing and taxonomy : Virology meeting room	Nicolas Sauvion & Barbara Jarausch
Database and website : Virology meeting room	Florin Oancea & Xavier Foissac

**10h30-10h45**      **Coffee break Floor 0**

**10h45-11h40**      **Practical sessions reports (max 10 min each)**

Triplex Realtime-PCR for grapevine phytoplasma: Ibolya Ember  
Typing of group V phytoplasmas : Dijana Skoric  
Typing of group X phytoplasmas : Jean-Luc Danet  
Typing of group XII phytoplasmas : Anne Fabre  
Insect typing and taxonomy : Renata Fialova

**11h40- 12h15 Research activities not related to SEE-ERAnet tasks**

**11h30-11h50** : Slobodan Krnjajić, IPPE- Department of Plant Pests :  
current research and prospective  
**11h50-12h10** : X. Foissac, Epidemiology of marginal chlorosis of strawberry  
**12h10-12h25** : S. Eveillard, *Ca. Liberibacters* and citrus HLB, an update

**12h30-13h30**      **Lunch at INRA restaurant**



**REPORTS & RELATED TOPICS**  
**Thursday 24 June Afternoon**  
**Virology meeting room**

**13h40 – 15h30            Activities not related and stolbur phytoplasma genetic diversity**

**13h40-14h00 : Invited speaker : Joel Renaudin**, Functional genomics and strategies to study *Spiroplasma citri* interactions with its plant host and insect vector

**14h00-14h20 : Invited speaker : Nathalie Bouvery**, Mollicute-insect hosts interactions : Cellular model and protein-protein interactions

**14h20-14h40: Invited speaker : Pascal Sirand-Pugnet** : Comparative genomics of Mollicutes

**14h40-15h00:** Xavier Foissac, Vmp1, a gene encoding a variable putative membrane protein of the stolbur phytoplasma. First variability studies in France, Italy, Czech republic, Lebanon and Azerbaijan.

**15h00-15h20 :** Jes Johannesen and Michael Maixner, VMP1-RFLP variation in Germany, sequence variations and geographic distributions in VMP1 and Stol-11

**15h20-15h40 :** Dijana Skoric, Bilateral visit of Martina Music to UMR-GDPP

**15h40-16h00 : Invited speaker : Renata Fialova** Typing of phytoplasma isolates from the Czech Republic

**16h00-16h20 :            Coffee break Floor 0**

**16h20 – 16h15 :            Group picture on stairs of IBVM hall**

**16h15-18h15 :            Working group reports (15 minutes + 5 min discussion)**

**16h15-16h20 Special adress by** Alain Blanchard, head of UMR-1090, Vice President of University of Bordeaux II, IOM chair-elect

**16h20-16h40** Typing of group V phytoplasmas : Sylvie Mal.-Maher

**16h40-17h00** Typing of group X phytoplasmas : Wolfgang Jarausch

**17h00-17h20** Typing of group XII phytoplasmas : Michael Maixner

**17h20- 17h40** Insect typing and taxonomy : Nicolas Sauvion

**17h40–18h00 Invited speaker : Daciana Papura** (UMR Plant Health, INRA-ENITA Bordeaux) : *Scaphoideus titanus* population genetics and bases for a phylogeographic study at the European scale.

**EUROPEAN PERSPECTIVES**  
**Friday 25 June**  
**Virology meeting room**

**9h15 – 9h45 Working group reports (20 minutes + 10 min discussion)**

**9h15-9h35** Database and website : Florin Oancea

**9h35-9h45** Discussion

**9h45- Formal propositions: bilateral and multilateral collaborations (10 min each)**

**9h45-9h55** Xavier Foissac :

- Epidemiology of Stolbur and Fruit tree phytoplasmas in Eastern Europe and Caspian Area – ECONET possible partners : France, Azerbaijan, Bulgaria, Romania, Czech Republic, Serbia, Croatia.
- Epidemiology of grapevine Yellows and survey for insect vector – Balaton Hungarian-French bilateral project. Submitted spring 2008
- Incidence and genetic diversity of phytoplasmas and spiroplasmas affecting agriculture in Northwestern governorates of Egypt : IMHOTEP Bilateral project being currently submitted with the Faculty of Agriculture, Kafrelsheikh University .

**9h40-9h50** Dijana Skoric :

- Israeli-Croatian bilateral project on the molecular epidemiology of grapevine phytoplasmas (under review, participants : Weintraub, Gera, Skoric, Seruga Music, Krizanac, Mikec, Budinscak).
- Cogito – France – Croatia : Epidemiology of Stolbur and Fruit tree phytoplasmas

**9h50-10h00** Sylvie Malembic-Maher

- Galileo Italian-French bilateral project : detection and diversity of FD Phytoplasma and related phytoplasmas of vineyard and wild eco-system in France and Italy. Submitted spring 2008
- Variable surface proteins in phytoplasmas of taxonomic group 16SrV.

**10h-00-10h10** Florin Oancea

- REGPOT 2009 – Use of phytoplasma collections for plant breeding.

**10h10-10h20** Other bi-lateral collaborations in place, being submitted or proposed

**10h20-10h50 Coffee break Floor 0**

**10h50-12h00                    Proposition of research topic to develop**

**10h40-10h55** Slobodan Krnjajic

- Current distribution of maize redness disease and impact on corn production
- New strain of *Ca. Phytoplasma ulmi* – distribution, epidemiology and pathogenicity

**11h10-12h10   FP7 – SEE-ERANET+ propositions**

- Renewal of SEE-eranet network on epidemiology (complementarity to COST proposal )
- Xavier Foissac (2009) : A specific project on Stolbur : MOLDEPHY : molecular determinants of phytoplasma pathogenicity and epidemic properties.
- Other propositions

**12h30-14h00 :                    Lunch at INRA restaurant**

**14h00-15h00 :**                    Open discussion regarding to projects and European perspectives  
Where are Visio-conference facilities ? Tests to be planned at fall 2008

**15h00-15h30 :**                    Projects of publications as outcomes of the SEE-ERANET network

**15h30-16h00 :**                    Participants conclusions  
Future ERANET milestones

**16h00-17h00                    End of the meeting & farewell coffee break floor 0**

**16h30-18h00                    Second visit** of Phytoplasma collection –greenhouses and insectarium  
& Laboratory visit for participants arrived on Wednesday and Thursday.