COST STSM Scientific Report - Ibolya Ember

COST Action: FA0807

COST STSM Reference Number: COST-STSM-ECOST-STMS-FA0807-150511-007737

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STSM Title: Identification methods and molecular characterization of endosymbionts and pathogens in *Cacopsylla pyri* vector of '*Candidatus* Phytoplasma pyri'

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Period: 15 - 24 May 2011 (10 days)

Identification methods and molecular characterization of endosymbionts and pathogens in *Cacopsylla pyri* vector of '*Candidatus* Phytoplasma pyri'

Background

At present there is no direct cure method either for the phytoplasmosis in the plant or for the control of the vectors with insecticides applied in the practice of crop management. The current knowledge suggests that the phytoplasma vector species belonging to Psyllids and Auchenorrhyncha are important insect hosts of pathogenic and endophytic bacteria as well. To analyse the microbial community within phytoplasma vector species can lead to the identification of novel species, potentially transmittable to host plants. Furthermore, in case the found bacteria are transmittable and not pathogenic; they could drive a protective effect on the plant due to the competitive displacement of the phytoplasma by competition for the same niche in the phloem cells.

Purpose of the visit

The aims of this STSM were to acquire identification method of PCR Denaturing Gradient Gel Electrophoresis (PCR-DGGE); to perform PCR-DGGE for determination of symbiotic microbial organism community of *Cacopsylla pyri, C. pyricola, C. pyrisuga* collected from PD infected orchards from Hungary and Italy. Perform analyses of Psyllids, Cicxiids and plants from potato fields of Hungary, Russia and Romania using PCR-DGGE. Furthermore carry out molecular analysis on the collected samples for determination of phytoplasma infection.

Description of the work carried out during the visit

Sampling and DNA extraction

Joint sampling was organized to collect insects in Italian pear orchards. Morphological identification of the collected Psyllids from Hungary and from Italy was performed. *Cacopsylla pyri*, *C. pyricola* and *C. pyrisuga* were selected for DNA extraction used CTAB method (Sambrook *et al.*, 1989) with slight modifications. The DNA extraction in case of potato Psyllids, Cixiids and plants (pear, potato, weeds) from Hungary was already done with LC Magna Pure, Tissue kit II (Roche) and CTAB method (Dair *et al*. 1997) (Table 1).

PCR amplification for DGGE

The total DNA mixture isolated from single individuals of insects and plants were used as template in PCR amplifications of 16S rRNA gene containing a 40bp GC clamp which insure that the fragment remains partially double-stranded during the DGGE. In total 80 samples were introduced for PCR and among these 70 samples were amplified by GC clamped, 16S rRNA bacterial primers. For DGGE 40 of positive samples resulted ~600bp long amplicon were selected. Altogether 40 samples were divided in two DGGE gels. For the first gel samples s from pear fields were assembled: 4 *Cacopsylla pyricola*, 7 *C. pyri,* 3 *C. pyrisuga* and 6 pears. On the second gel, samples from potato field were grouped: 4 Psyllids (2 *Bactericera nigricornis,* 2 *Cacopsylla* sp.), 8 Cixiids (2 *Reptalus*

panzeri 2 *R. quinquecostatus,* 2 *Hyalesthes obsoletus* and 1 *Pentastiridius* sp.) and 6 potatoes, 1 tomato, 1 *Conium maculatum.*

DGGE

For Denaturing Gradient Gel Electrophoresis 7% of polyacrylamide gel with a 40% and 60% denaturant concentration of urea and formamide were prepared. The same size DNA fragments with different sequences were differentiated based on their diverse denaturation (melting) profile. The running was done within 15 hours at 90V. The gels were stained with SYBR-Green, then the visualisation and image documentations were performed followed by cutting 73 of DGGE bands from the 2 gels.

PCR re-amplification for sequencing

The cut and eluted products were re-amplified with 16SrRNA primers without GC clamp. In total 12 amplicons were appropriate for sequencing (Fig.1). All the amplified samples were sent for sequencing. The obtained 12 sequences were compared with the sequences in database using BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST). According to the BLAST analysis (Table 2), the DNA recovered from one band belonged to '*Ca*. Liberibacter europaeus', and one band was related to an uncultured bacterium previously described in association with the psyllid *Diaphorina citri*. Furthermore, the amplicons obtained from three bands were referable to symbionts of the genus *Arsenophonus,* other three bands were related to a symbiotic *Rickettsia*, and the products of further three bands were allied to bacteria of the genus *Paracoccus*.

PCR/RFLP to identification of phytoplasma

Psyllid and pear samples from Hungary and Italy were tested for phytoplasma infection using P1/P7 and FO1/RO1 primers. The RFLP analysis, using *Ssp*I and *Rsa*I restriction enzymes, of the positive samples resulted Pear Decline (16SrX-C) phytoplasma in 4 *Cacopsylla pyri*, 2 *C. pyricola* and 3 pears samples from Hungary.

References

Daire X., Clair, D., Reinert, W., & Boudon-Padieu E. (1997). Detection of grapevine yellows pytoplasmas belonging to elm yellows group and to the Stolbur subgroup by PCR amplification of non-ribosomal DNA. European Journal of Plant Pathology, 103:504-507.

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Raddadi N., Gonella E., Camerota C., Pizzinat A., Tedechi R., Crotti E., Mandrioli M., Bianco P.A., Daffonchio D and Alma A. 2010. '*Candidatus* Liberibacter europaeus' sp. nov. that is associated with and transmitted by the psyllid *Cacopsylla pyri* apparently behaves as en endophyte rather than a pathogen. Environmental Microbiology 13(2):414–426.

www.ncbi.nlm.nih.gov/BLAST

Table 1. List of plant and insect samples

Species	Location	Number of tested samples	Sampling date
Samples from pear orchard			
Pyrus communis	Hungary	8	12.05.2011
Cacopsylla pyri	Hungary	10	12.05.2011
Cacopsylla pyri	Italy	7	16.05.2011
Cacopsylla pyricola	Hungary	14	12.05.2011
Cacopsylla pyricola	Italy	8	16.05.2011
Cacopsylla pyrisuga	Hungary	11	12.05.2011
Cacopsylla pyrisuga	Italy	2	16.05.2011
Samples from potato field			
Beta vulgaris	Russia	1	12.07.2010
Capsicum annuum	Hungary	1	31.08.2010
Conium maculatum	Romania	1	27.05.2010
Convolvulus arvensis	Romania	1	27.05.2010
Cuscuta sp.	Russia	1	14.07.2009
Lycopersicon esculentum	Romania	2	23.07.2010
Lycopersicon esculentum	Hungary	3	13.08.2010
Petroselinum crispum	Hungary	2	13.08.2010
Solanum tuberosum	Romania	5	18.06.2009
Solanum tuberosum	Russia	7	14.07.2009
Solanum tuberosum	Hungary	4	27.07.2010
Bactericera nigricornis	Romania	1	16.06.2009
Bactericera nigricornis	Russia	2	01.07.2009
Bactericera nigricornis	Hungary	2	29.06.2010
Bactericera sp.	Hungary	2	29.06.2010
Cacopsylla sp.	Hungary	5	18.06.2010
Psylla sp.	Romania	1	31.07.2008
Trioza sp.	Russia	1	08.06.2009
Trioza sp.	Romania	3	28.06.2010
Trioza sp.	Hungary	1	16.07.2010
Empoasca pteridis	Russia	1	12.07.2010
Eupteryx atropunctata	Russia	1	19.08.2009
Hyalesthes obsoletus	Romania	2	01.07.2008
Hyalesthes obsoletus	Russia	3	12.07.2010
Macrosteles laevis	Russia	1	19.08.2009
Macrosteles sp.	Russia	1	29.08.2009
Neophilaenus sp.	Russia	1	12.07.2010
Pentastiridius sp.	Russia	2	12.07.2010
Plagiognathus sp.	Russia	1	19.08.2009
Psammotettix alienus	Russia	1	13.08.2008
Reptalus cuspidatus	Russia	2	12.07.2010
Reptalus melanochaetus	Russia	1	12.07.2010
Reptalus panzeri	Romania	4	16.06.2008
Reptalus quinquecostatus	Romania	9	09.06.2009

Figure 1. Image of the excised bands that were successfully sequenced

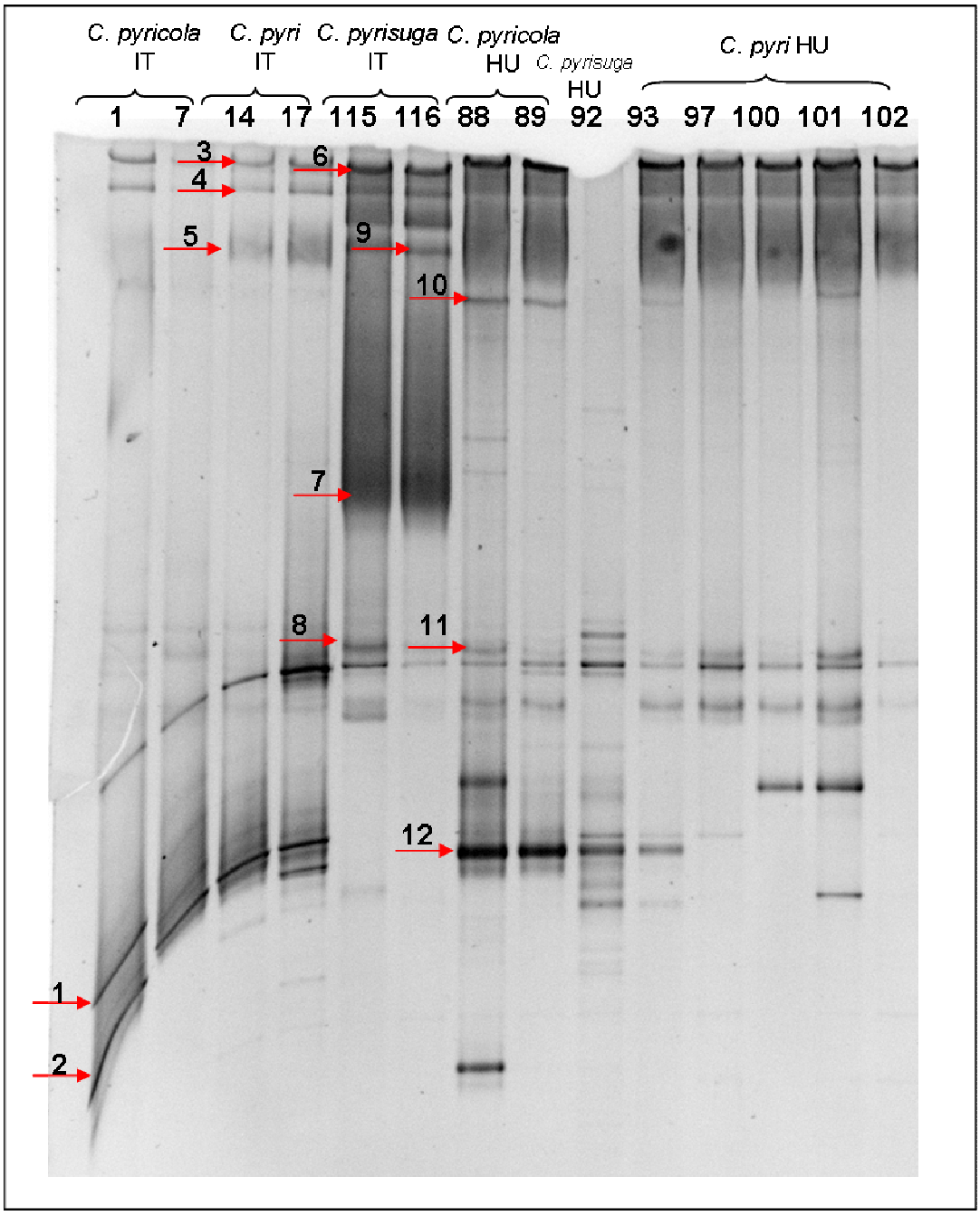

Table 2. Results of sequencing of viable DNA obtained from the DGGE bands

Band ID	Closest relative Accession Number	Closest relative name	Similarity %
1	FN678792	*Ca.* Liberibacter europaeus	99% (499/500 bp)
2	GU553034	Uncult. bacterium of *D. citri*	99% (515/519 bp)
3	DQ314776	*Arsenophonus* sp.	99% (533/540 bp)
4	AF286125	*Arsenophonus* sp.	99% (537/546 bp)
5	AF286125	*Arsenophonus* sp.	99% (533/540 bp)
6	FM955311	*Rickettsia* sp.	99% (511/517 bp)
7	FM955311	*Rickettsia* sp.	99% (515/518 bp)
8	FM955311	*Rickettsia* sp.	99% (516/519 bp)
9	FM955311	*Rickettsia* sp.	99% (515/518 bp)
10	JF792359	*Paracoccus* sp.	99% (510/514 bp)
11	DQ659042	*Paracoccus* sp.	100% (536/526)
12	GQ169043	*Paracoccus* sp.	100% (520/520 bp)