Minutes of the training school on "Molecular Tools to Identify Leafhopper and Planthopper Vectors of Phytoplasmas"

Monday, November 5th 2012 (Ivrea).

Domenico Bosco opened the training school by welcoming the participants and giving an overview of the course activities. The theoretical session held in Ivrea on Monday and Tuesday was organized by the section of Entomology and Zoology, DISAFA, Università degli Studi di Torino, in collaboration with the Istituto per la Protezione delle Piante (IPP), CNR, Portici, Napoli. The session was in the frame of the PRIN project "Identification of species and intra-specific variants of sap-sucking and parasitoid insects with molecular markers" granted by the Italian Ministry of the Education and Research (MIUR). The speeches were made by the COST teachers Domenico Bosco and Sabrina Bertin from DISAFA, and Maurilia Monti, Paolo Pedata and Giuseppe Parrella form IPP.
The speakers firstly gave a comprehensive overview of the insect systematic and taxonomy, facing the different approaches and theories for the organism classification and identification. Particular attention was paid to the role of DNA markers as a support of the traditional morphology-based taxonomy and the importance of a multi-disciplinary approach (the "integrative taxonomy") for the delimitation of the species boundaries. The concept of DNA-barcoding and the procedure for sequence submission in the Barcoding of Life Database (BOLD) have been deeply described.
Secondly, the speakers presented concepts and aims of the main molecular markers used for species identification (mitochondrial DNA, ribosomal DNA, protein-coding genes) as well as population genetics (RAPD, microsatellites). Moreover, insights into DNA preparation and conservation as well as an introduction of the main approaches for phylogeny inferences were given. A practical demonstration of DNA-sequences handling, sequence comparison (in BLAST and ClustalW workbenches) and phylogenetic analysis in MEGA5 was showed.

Tuesday, November 6th 2012 (Ivrea).

The speakers presented applications of molecular markers for insect species identification, biotypes differentiation and species-complex description. Those works focusing on the molecular identification of the planthopper species *Hyalesthes* and *Reptalus* (Cixiidae family) and the phylogeny of the leafhopper tribe Empoascini (Cicadellidae family) were of particular interest for the ascertained/potential role of these insects in phytoplasma transmission. These applications represented also the base for the practical session of the course.
The Bio-Rad Company presented the High Resolution Melting PCR as a new tool for the differentiation of closely-related samples. Its potential application for insect taxonomy was pointed out.
The theoretical session closed with a comprehensive overview of the Auchenorrhynca morphology and taxonomy, with a particular focus on Cixiidae and Deltocephalinae. Luca Picciau described the morphological features of wings, head, thorax, legs and abdomen useful for the classification at family/subfamily and genus level. Afterward, detailed information on male genitalia morphology were provided to achieve the identification of the main species involved in phytoplasma transmission.

Wednesday, November 7th 2012 (Grugliasco).

The practical course in the DISAFA labs was guided by Sabrina Bertin and Rosemarie Tedeschi. Total DNA extraction from *Hyalesthes*, *Reptalus* and *Empoasca* specimens was preformed. The CTAB-based protocol, suitable also for phytoplasma DNA isolation, was employed. The participants worked in three different groups. The DNA samples were measured at Nanodrop spectrophotometer, in order to check the quality and the amount of the extracted DNA.
Two different PCR assays amplifying the subunit I of the mitochondrial cytochrome-*c*-oxidase (COI) have been performed on *Hyalesthes* species (*H. obsoleuts*, *H. luteipes* and *H. scotti*) and *Reptalus* species (*R. quinquecostatus*, *R. cuspidatus*, *R. panzeri* and *R. melanochaetus*), respectively. The PCRs provided the amplification of a ~ 900bp fragment of the COI gene.
The afternoon session was devoted to the practical identification of cixiids and cicadellids by means of morphological features. Based on the information received the day before, the participants were in condition to perform the recognition of the main distinctive characters of the species *Hyalesthes obsoleuts*, *H. luteipes* and *H. scotti*; *Reptalus quinquecostatus*; *Euscelidius variegatus*. Thanks to a stereo-microscope connected to a camera, the participants could firstly follow the dissection procedures performed by Luca Picciau and see the diagnostic characters. Later, the participants were allowed to try to dissect and recognize the samples by themselves at different stereo-microscopes.

Thursday, November 8th 2012 (Grugliasco).

A PCR assay amplifying the ITS2 ribosomal region was performed on Empoascini specimens belonging to the genera *Empoasca*, *Asymmetrasca* and *Jacobiasca*. The electrophoresis run of the PCR products was carried out in the afternoon and the gel pattern provided an evident differentiation of the three genera by means of a different ITS2 length. The experiment allowed the participants to assign to the correct genus the specimens whose DNA was blindly extracted the day before without the previous morphological identification.
The COI-PCR products from *Hyalesthes* samples were digested with the restriction enzyme *Taq*I. The different restriction sites on the COI products allowed to discriminate the species *H. obsoleuts*, *H. luteipes* and *H. scotti* by means of different electrophoretic profiles. The RFLP gel was prepared by mixing normal agarose (Seakem LE) with Metaphore agarose, specific for a better resolution of small DNA fragments. The mixed gel was 2% concentrated and was allowed to run at low voltage (70V) in order to ensure reliable RFLP patterns. These devices avoid the undesirable use of polyacrylamide in routine gel preparation.
The 900bp COI-PCR products from *Reptalus* samples were separated on an agarose gel and afterward purified by means of the PureLink™ Purification Kit, Invitrogen. The samples clearly showing a unique 900bp gel band were purified directly from the PCR reaction mix. Those samples that showed a-specific amplified fragments were purified by excising the 900bp band from the gel, in order to minimize the contamination by uninformative PCR products. Purification processes from PCR mix and gel-band excision were both performed following the manufacturer's instructions. At the end of the procedures, the participants received useful instructions for sending the purified products to a sequencing service.
The evening was spent in the “Museo Regionale di Scienze Naturali” of Torino. The participants had the opportunity to visit different historical Entomological collections pertaining the University of Turin and Museo Regionale di Scienze Naturali (i.e. Spinola collection).

Friday, November 9th 2012 (Grugliasco).

A computer session was set up to interact with the main programs that can be useful in developing molecular markers for species identification and insect taxonomy. A computer per each participant was available and the lesson was guided by the pilot experiments made by Sabrina Bertin. The lesson dealt with the following topics:

- editing of raw sequences in Chromas programs; assembling forward and reverse sequences in a consensus sequence in ChromasPro program;
- comparing and aligning sequences in ClustalW and ClustalX programs;
- simulating RFLP assays by means of *in silico* enzyme digestion. Different programs have been used (WebCutter, REPK Restriction Enzyme Picker Online). The main elements necessary for the choice of the best enzymes discriminating among different samples have been discussed.

The course closed with a brief final discussion of the obtained results.

The participants received a copy of the protocols used in the practical session and a CD including the Power Point presentations of the theoretical session. Protocols and abstracts of the theoretical session are also available in the COST Action FA0807 web site: (http://www.costphytoplasma.eu/WG2.htm)