

NOTE: A Comparison of Molecular Diagnostic Procedures for the Detection of Aster Yellows Phytoplasmas (16Sr-I) in Leafhopper Vectors

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Different molecular procedures were compared for the detection of aster yellows phytoplasmas (AYP) in the leafhopper vectors *Macrostelus quadripunctulatus* (Kirschbaum), *Euscelidius variegatus* (Kirschbaum) and *Euscelis incisus* (Kirschbaum). Polymerase chain reaction (PCR) with universal and group-specific primers designed on the 16S-rDNA sequence was most sensitive in nested assays. A dot-blot procedure with an oligoprobe designed on the 16S-rDNA was less sensitive and consistent to detect phytoplasmas in total insect DNA, but consistently detected amplicons from direct PCR. The dot-blot assay with a probe based on a phytoplasma plasmid sequence detected AYP in most vector specimens and did not react with DNAs from leafhoppers infected by flavescence dorée and psyllids infected by apple proliferation phytoplasmas. This last assay is almost devoid of contamination risks, faster and cheaper compared to PCR, therefore it has to be preferred for field-scale analysis of leafhopper populations.

KEY WORDS: *Macrostelus quadripunctulatus*; *Euscelidius variegatus*; *Euscelis incisus*; phytoplasmas; molecular detection.

Phytoplasmas are phloem-inhabiting prokaryotes, transmitted by phloem-sucking insects and associated with many diseases of several plant species. Many yellows diseases of ornamentals (such as daisy, primrose, gladiolus, cyclamen) and vegetables (lettuce, broccoli, etc.), collectively called aster yellows (AY), are associated with phytoplasmas belonging to the 16Sr-I genetic group (AYPs).

AYP is persistently transmitted by different species of leafhoppers belonging to the family Cicadellidae. The detection of phytoplasmas in the insect vectors is useful to identify new vector species and to monitor infection pressure under field conditions. AY affect a large number of plant species but so far only a limited number of leafhopper vectors have been identified.

Moreover, sudden and unforeseen epidemics of AY frequently occur both in the open field (*e.g.* lettuce) and in protected crops (*e.g.* ornamentals). The presence of leafhopper vectors alone is not a reliable index of infection risks; the numbers of infective vectors are a better indication. Moreover, AYP vector species are low-density pests that do not cause direct damage to host plants, requiring control measures. For these reasons the monitoring of leafhopper populations for the presence of phytoplasma is essential for rational control or prevention of the disease (4).

We compared different molecular procedures, direct and nested PCR, and hybridization with chromosomal and extrachromosomal probes, for the detection of AY phytoplasmas in the most common leafhopper vec-

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tor species in Italy: *Macrostelus quadripunctulatus* (Kirschbaum), *Euscelidius variegatus* (Kirschbaum) and *Euscelis incisus* (Kirschbaum) (2). A fast, reliable and low-cost hybridization technique is suggested for field-scale analysis of leafhopper populations.

Potted plants of *Chrysanthemum carinatum* (Schousboe) infected with an AYP strain belonging to the 16SrI-B subgroup, previously characterized and named chrysanthemum yellows (CY) (12), were used as source plants. Healthy colonies of leafhoppers were reared on potted oat (*M. quadripunctulatus* and *E. variegatus*) or white clover (*E. incisus*) plants kept inside plexiglas and nylon cages (100 × 40 × 50 cm) in climate-controlled chambers at 25°C with a 16L:8D photoperiod. The phytoplasma-free status of insects was tested as follows: in each DNA extraction procedure of infected leafhoppers, two samples from healthy colonies were included and then analyzed by polymerase chain reaction (PCR). Healthy 4th–5th instar nymphs were taken from the colonies and group-caged for one week on CY-infected source plants. Source plants were 20–25 cm high at the time of acquisition and had been inoculated by insects 20 days previously. Leafhoppers were then transferred onto healthy oat plants to complete latency. Twenty-five days after the beginning of the acquisition, the adults were sampled and DNA was extracted from single adults. Under the same experimental conditions, different AYP transmission efficiencies have been reported for *M. quadripunctulatus* and *E. variegatus*: 100% and 30%, respectively (13). AYP transmission efficiency of *E. incisus* is similar to that of *E. variegatus* (unpublished results).

Total DNA was extracted from 116 *M. quadripunctulatus*, 126 *E. variegatus* and 38 *E. incisus* single adults as described in Bosco *et al.* (3). All samples were analyzed with PCR while a large sub-set (103, 117 and 34 for *M. quadripunctulatus*, *E. variegatus* and *E. incisus*, respectively) were tested in dot-blot of total insect DNA. For PCR analyses, aliquots of insect DNAs were amplified using the following phytoplasma-specific reagents: R16F2/R2 (9) primer pair in direct PCR assays, and the internal one R16F1/R1(I) (8) in nested PCR assays. A chromosomal oligoprobe (CYS2), designed on

the sequence of AYP16S rDNA (7), was 3' end labeled with digoxigenin according to the manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, IN, USA), and a 235 bp plasmid-derived probe (PK6/K8) was labeled with digoxigenin by PCR driven by CK6/CK8 plasmid-derived primer pair (5). CYS2 oligoprobe, internal to the R16F2/R2 amplicon, was used for hybridization assays of both PCR amplicons and of total insect DNA, whereas PK6/K8 was used to probe total insect DNA only. PCRs were performed according to Bosco *et al.* (3), whereas for dot-blot hybridization assays 20 µl of the PCR amplicon or of total insect DNA (out of 50 µl) was spotted *in vacuo* onto a positively charged nylon membrane (Roche). Hybridization was performed at 45°C with CYS2 oligoprobe or overnight with PK6/K8 probe. Two stringency washings were performed at 40°C each for 15 min with 0.5 × SSC, 0.1% SDS, for both probes. Detection was performed with the chemiluminescent substrate CDP-Star (Roche), according to the manufacturer's instructions. Samples of *Scaphoideus titanus* Ball and *Cacopsylla melanoneura* (Förster) infected with flavescence dorée (FD, 16Sr-V genetic group) and apple proliferation phytoplasmas (AP, 16Sr-X genetic group) were included in the hybridization assays to check the specificity of the diagnosis. *S. titanus* samples were collected in FD-infected vineyards of the Piemonte Region (northwestern Italy) and *C. melanoneura* samples were collected in AP-infected apple orchards of the same region.

Results of direct and nested PCR assays of the three leafhopper species are summarized in Table 1. The three detection procedures provided significantly different results in the different vector species (chi-square tests, $P < 0.05$). As expected, nested PCR was the most sensitive detection method, enabling the detection of CYP in 89–93% of the samples, whereas direct PCR assay detected CYP in 60–66% of the tested leafhoppers. However, nested PCR is expensive, time-consuming and has serious contamination risks; moreover, under our experimental conditions, comparisons of detection and transmission results (13) still indicated the presence of approx. 10% false negatives in *M. quadripunctulatus*. Dot-blot hybridization of the amplicons

from direct PCR detected CYP in 71–83% of the leafhoppers, thus improving the sensitivity of the direct PCR assay. This increase was statistically significant for *M. quadripunctulatus* and *E. variegatus*, but not for *E. incisus*. Dot-blot hybridization of the amplicons from direct PCR has been reported to increase the detection of the target phytoplasma DNA in a plasmid-based assay (5). Detection of AYP provided similar results for the three vectors, suggesting that all the leafhopper species have similar efficiency in

acquiring CY, even though they show different transmission rates. Results of dot-blot hybridizations of total insect DNA using chromosomal and plasmid-derived phytoplasma-specific probes are shown in Table 1. DNA from FD- and AP-infected insects never reacted with either probe, even at low stringency conditions. The efficiency of the plasmid-derived PK6/K8 probe was surprisingly high, and CY was detected in 83–85% of *M. quadripunctulatus* and *E. variegatus* samples (Fig. 1).

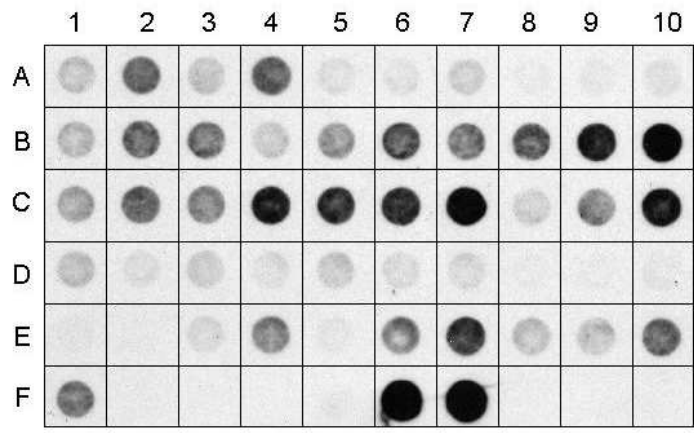


Fig. 1. Dot-blot hybridization of total DNA from *Macrosteles quadripunctulatus* (A1-C7) and *Euscelidius variegatus* (C8-F1) with the plasmid-derived PK6/K8 probe. F2, FD-infected *Scaphoideus titanus*; F3, AP-infected *Cacopsylla melanoneura*; F4, healthy *M. quadripunctulatus*; F6 and F7; CY positive controls.

The efficiency of hybridization with both probes was not influenced by the size of the vector, *M. quadripunctulatus* being much smaller than the two other species (DNAs from single insects were resuspended in the same volume regardless of the species). Different titers of phytoplasmas in the different vector species cannot be ruled out (1); however, our dot-blot assay was not designed to provide quantitative results. Hybridization with the PK6/PK8 probe was much more sensitive than that with chromosomal CYS2 (chi-square tests, $P < 0.05$) in all three leafhopper species and even more sensitive

than the direct PCR in *M. quadripunctulatus* and *E. variegatus*. The lower sensitivity of the direct PCR assay with R16F2/R2 vs hybridization with the PK6/K8 probe could be explained by (i) the presence of polymerase inhibitor in the insect DNA preparation, although in previous experiments inhibitors were found only in DNA preparations from stored *M. quadripunctulatus* and *E. incisus* (10); and (ii) the different target DNAs for hybridization (plasmid DNA) and amplification (chromosomal DNA). The higher sensitivity of the PK6/K8 probe compared with CYS2 could be due to the higher copy number of target

sequence and/or to the different labeling method: PCR incorporation vs 3' end labeling. Kuske and Kirkpatrick (6) also experienced a higher sensitivity of the extrachromosomal vs chromosomal probe in detecting dwarf and severe aster yellows phytoplasmas in periwinkle plants. The use of the plasmid target to detect AYPs in insect vectors increases sensitivity and should not provide false negative samples because such plasmid sequences have been repeatedly and constantly found in AYP strains from different geographical areas (3,5). On the other hand, false negative results following a plasmid-based detection cannot be completely ruled out since the presence of deletion mutant plasmids has been reported in a closely related phytoplasma strain (11). This deletion has been tentatively associated with the insect host specificity.

The PK6/K8 probe proved to be quite specific to group I phytoplasma also under low stringency conditions, and we therefore suggest this assay for large-scale analysis of AY phytoplasmas in vector insects. The specificity of such an extrachromosomal sequence has been debated (5,7,14), but in our hands PK6/K8 probe never

provided hybridization signals with DNAs from FD and AP phytoplasmas. However, in PCR assays driven by the flanking primers CK6/CK8, a few amplicons could be obtained from AP-infected psyllids (3). Hybridization is far less expensive than PCR and almost devoid of the risk of contamination. Ninety-six insects can be analyzed in a single 10×13 cm membrane using an *in vacuo* manifold, and more than one membrane can be processed at the same time.

To monitor the presence of phytoplasmas in field populations of vector leafhoppers, fresh, acetone- or ethanol-stored insects can be used (3), and control measures can be applied only when phytoplasma-positive leafhoppers are identified. Although the identification of phytoplasma in the leafhopper does not provide definite evidence of its vector capability (15), the identification of a phytoplasma in a species recognized as a vector is a very reliable indication of transmission risk. When phytoplasma-infected plants are already present in the crop, the presence of leafhopper vectors, infected or not, should be excluded.

TABLE 1. PCR and hybridization assays of single leafhoppers exposed to CY phytoplasma infection (positive/tested). All the samples in the two last columns tested positive in (direct or nested) PCR assays.

Leafhopper species	Direct PCR (R16F2/R2) (%)	Nested PCR (R16F1/R1) (%)	Hybridization of F2/R2 amplicons with CYS2 probe (%)	Hybridization of total DNA with CYS2 probe (%)	Hybridization of total DNA with PK6/K8 probe (%)
<i>Macrostelus quadripunctulatus</i>	77/116 (66.4) a	103/116 (88.8) b	91/116 (78.4) c	33/103 (32.0) A	88/103 (85.4) B
<i>Euscelidius variegatus</i>	72/126 (61.1) a	117/126 (92.9) b	105/126 (83.3) c	46/117 (39.3) A	97/117 (82.9) B
<i>Euscelis incisus</i>	23/38 (60.5) a	34/38 (89.5) b	27/38 (71.1) a	7/34 (20.6) A	18/34 (52.9%) B

Within rows, percentages followed by the same letter do not differ significantly (chi-squared paired comparisons for all possible combinations; $P < 0.05$).

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