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Detection and molecular characterization of phytoplasmas in the planthopper *Metcalfa pruinosa* (Say) (*Homoptera: Flatidae*) (*)

Since 1980 Italian entomologists had to deal with a new pest: the neartic flatid *Metcalfa pruinosa* (Say) was found nearby Treviso (Veneto) (first report: Zangheri and Donadini, 1980) and spread widely out in north-eastern (Duso, 1984; Duso and Pavan, 1988; Cravedi, 1989), northwestern (Arzone and Arnò, 1989) and central Italy (Santini, 1991; Bin *et al.*, 1993).

Native of an area that spaces from eastern-Canada to Brasil (Dean and Bailey, 1961; Wilson and McPherson, 1981), *M. pruinosa* is extremely polyphagous. Due its great adaptability it became an important pest in Italy, infesting both forest and fruit trees, bothering ornamental plant growers, damaging vineyards and feeding on a huge number of spontaneous and cultivated plants (Duso and Pavan, 1988; Santini, 1991). While dense populations of nymphs cause stunting of plant shoots, adults are responsible for a large production of honeydew which promotes the development of sooty mould. This kind of damage has increasingly been observed in vineyards (Duso, 1984).

Northern Italy is not only a promised land for neartic flatids but also for plant pathologists and epidemiologists: it is a long time since domestic and foreign researchers have been focusing phytoplasma infections in Italian orchards. Thus, severe and minor diseases in fruit trees and grapevines have been associated with genetically diverse phytoplasmas (Poggi Pollini and Giunchedi, 1992; Bertaccini *et al.*, 1994; Prince *et al.*, 1993; Lee *et al.*, 1995) whose characterization was allowed thanks to classifications based on RFLP patterns of conserved ribosomal-RNA sequences (Schneider *et al.*, 1993; Lee *et al.*, 1993; Namba *et al.*, 1993) and supported by broad phylogenetical studies (Seemüller *et al.*, 1994; Gundersen *et al.*, 1994).

Molecular screening for detection of phytoplasmas in *M. pruinosa* therefore is basic for further research on its candidature as potential vector of phytoplasmas.

M. pruinosa adults were collected during summer 1994 and 1995 both on weeds and in orchards of northern Italy (Veneto, Emilia-Romagna). Following a modified DNA extraction procedure previously described (Goodwin *et al.*, 1994), single insects were micro-ground in sterile Eppendorf tubes with 200 µl TE buffer by means of sterile sand, boiled for 3 min and briefly centrifuged at 14,000 rpm in an Eppendorf microfuge; 1 µl of 1:50 sterile-water-diluted supernatant was used in PCR assays; a negative control without insect was obtained for each extraction series following the same procedure.

Nested-PCR assays were carried out with two sets of universal primer pairs: R16F1/R0 followed by R16F2/R2. Further 16SrRNA phytoplasma group-specific primer pairs were used in nested-PCR assays: R16(I)F1/R1, R16(III)F2/R1, R16(V)F1/R1, R16(X)F1/R1 (Lee *et al.*, 1994, 1995). Phytoplasma control strains were AY (Maryland aster yellows, Lee and Davis, 1988), CPh (clover phyllody, L.N. Chiykowski, Agriculture Canada, Ottawa, Ontario, Canada *via* I.-M. Lee), IPVR (Italian periwinkle virescence, Davis *et al.*, 1992), CX (B.C. Kirkpatrick, University of California, Davis, CA, USA *via* I.-M. Lee), EY1 (elm yellows, W.A. Sinclair, Cornell University, NY, USA *via* I.-M. Lee), AP (apple proliferation, L. Carraro, Università di Udine, Italy). PCR products were analyzed by electrophoresis through a 1% agarose gel and stained in ethidium bromide; DNA bands were visualized with an UV transilluminator.

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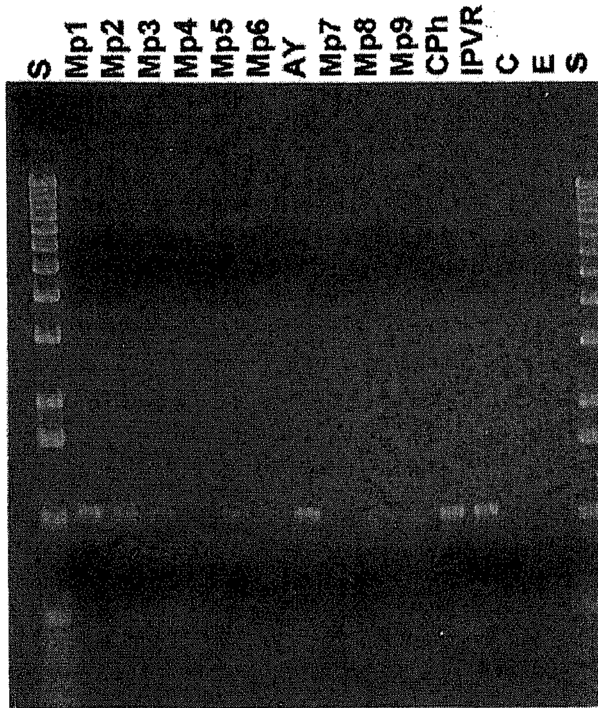

Fig. 1 - Agarose gel of nested polymerase chain reaction (PCR) of phytoplasma 16S rDNA sequences amplified from some of the *M. pruinosa* samples and from control phytoplasmas using primers R16(I)F1/R1. Samples Mp 1 to 9, *M. pruinosa*; AY, Maryland aster yellows; CPh, clover phyllody; IPVR, Italian periwinkle virescence; C, negative control without insect; E, water control; S, marker 1Kb DNA ladder, fragment sizes in kb from top to bottom: 12.2, 11.1, 10.1, 9.1, 8.1, 7.1, 6.1, 5.0, 4.0, 3.0, 2.0, 1.6, 1.0, 0.5, 0.3, 0.2, and 0.1.

Nested-R16F2/R2 products showed characteristic 1.2 kb DNA bands although not necessarily strong for *M. pruinosa* samples (data not shown). Further group-specific nested-PCR assays provided clear results. Phytoplasma control strains were all positive and the negative control without insect was always negative. While the totality of single *M. pruinosa* samples did not show amplification using 16S rRNA group-III and group-V primer pairs (data not shown), samples were positive to primer pair R16(X)F1/R1 specific to AP and related phytoplasma strains (Lee *et al.*, unpublished) and to primer pair R16(I)F1/R1 specific to AY and related phytoplasmas. R16(I)F1/R1 yielded amplification of specific 1.1 kb DNA fragments (Fig. 1, showing part of the 25 insect samples primed). The rate of positive samples was about 40%. Due to the low number of insects sampled, infection rates should not be considered statistically significant.

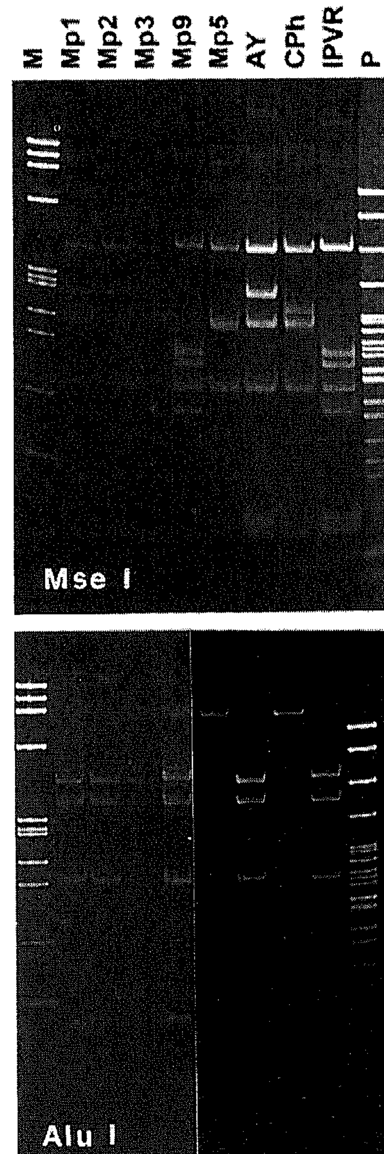

Fig. 2 - Polyacrylamide 5% gels showing the restriction fragment length polymorphisms of 1.1 kb phytoplasma 16S rDNA fragment amplified from different *M. pruinosa* and from phytoplasma controls. Sample abbreviations are as in Fig. 1, enzymes used are at the bottom of each gel. P, marker pBR322 *Msp*I digested; fragment sizes in base pairs from top to bottom: 622, 527, 404, 307, 242, 238, 217, 201, 190, 180, 160, 147, 123, 110, 90, 76, 67, 34, 26, 15, 9. M, marker ϕX174 *Hae*III digested; fragment sizes in base pairs from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72.

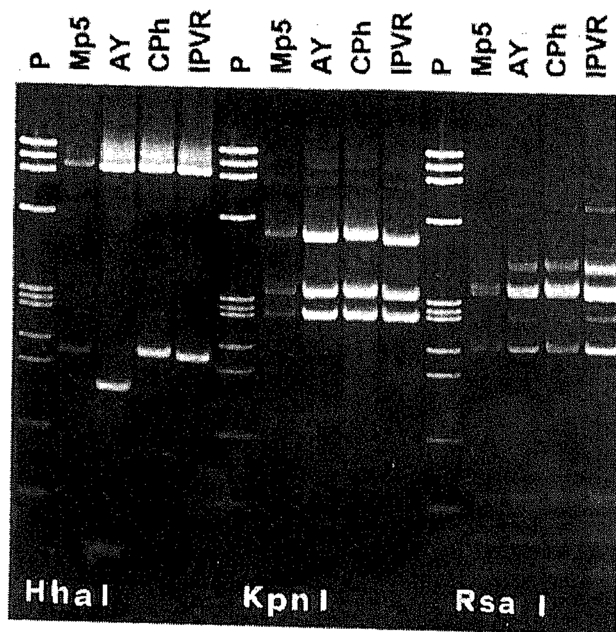

Fig. 3 - Polyacrylamide 5% gel showing the restriction fragment length polymorphisms of phytoplasma 16S rDNA fragments of 1.1 kb amplified from sample Mp5 in comparison with control phytoplasmas. Sample abbreviations are as in Fig. 1, enzymes used are at the bottom of the gel. M, marker ϕX174 *Hae*III digested; fragment sizes in base pairs from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72.

Restriction Fragment Length Polymorphism (RFLP) analysis of amplified R16(I)F1/R1 products with restriction enzymes *Mse*I and *Alu*I disclosed three different RFLP patterns after electrophoresis through a 5% polyacrylamide gel (Fig. 2). Reference strains for classification were AY1=16SrI-B, Maryland aster yellows; CPh=16SrI-C, clover phyllody and IPVR=16SrI-G, Italian periwinkle virescence (Lee *et al.*, 1993; Vibio *et al.*, 1996).

Although a pattern identical to 16SrI-G (IPVR and related strains) has been found (Fig. 2, Mp9), patterns 16SrI-B (AY1 related) seem to be predominant within the sampled insects (Fig. 2, Mp1, Mp2, Mp3 and data not shown).

RFLP pointed out a third unexpected pattern (Fig. 2, Mp5). Since the R16(I)F1/R1 amplification product clearly appeared as a specific 1.1 kb fragment (Fig. 1) and since similar patterns have been observed in investigations on *Scaphoideus titanus* Ball (Danielli *et al.*, unpublished), further RFLP analyses became worthwhile. Fig. 3 shows the R16(I)F1/R1 patterns of sample Mp5 after restriction with *Kpn*I, *Rsa*I and *Hha*I in comparison to AY subgroup control strains (16SrI-B, 16SrI-C, 16SrI-G). While identical *Kpn*I patterns confirm that this phytoplasma belongs to 16S rRNA group I in consistence with PCR results, *Mse*I, *Alu*I and *Rsa*I restriction enzymes clearly distinguish its singularity. *Hha*I polymorphism appears similar to 16SrI-C subgroup (CPh) and *Mse*I and *Alu*I patterns are referable to a 16SrI-C pattern devoiding bands, a relation within this subgroup might be suggested hence.

Presence in *M. pruinosa* of diverse phytoplasmas belonging to different groups (apple proliferation and related strains; aster yellows and related strains) and subgroups (Maryland aster yellows, 16SrI-B; Italian periwinkle virescence, 16SrI-G and a previously undescribed 16SrI pattern) was outlined. No mixed infection was detected. It is important to emphasize that presence of a phytoplasma in an insect does not necessarily involve its transmission to plants (Vega *et al.*, 1993). Further investigations are necessary to clarify the role of *M. pruinosa* as phytoplasma vector.

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