# PCR/RFLP-based method for molecular characterization of 'Candidatus *Phytoplasma prunorum*' strains using the *aceF* gene.

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## Abstract

New molecular typing tools for phytoplasmas belonging to the 16SrX phytoplasma group have recently been developed based on the non-ribosomal genes *aceF*, *pnp*, *imp*, and *SecY*. In the present work we chose to perform a PCR-RFLP method based on the *aceF* gene. This genetic marker had previously shown high variability among strains of the 16SrX group, moreover, it had allowed for the differentiation of French hypovirulent 'Candidatus *Phytoplasma prunorum*' strains from virulent ones.

Most of the stone fruit samples were collected in north-east Italy, although a few samples from Bosnia and Herzegovina, and Turkey were also included in the work to explore variability. French hypovirulent and virulent strains, one Azerbaijan strain and 'Ca. *P. prunorum*' strains maintained in periwinkles were used as reference strains. Some of the Italian samples were not collected in the field and they became infected by *Cacopsylla pruni* under controlled conditions.

Sequencing of the *aceF* gene was performed on some of the samples tested and based on the alignment, a few restriction enzymes were selected for 'Ca. *P. prunorum*' strain differentiation. Nested PCR was performed using previously developed primers on all samples and RFLP analyses were carried out with *Bpi*I, *Hae*III and *Tsp*509I enzymes. *Bpi*I and *Hae*III enzymes generated two different profiles, one profile was undigested and the second one constituted by two different fragments. The *Tsp*509I enzyme enabled three different pattern types to be distinguished. Combining the results obtained with the three restriction enzymes, it was possible to distinguish between the 'Ca. *P. prunorum*' strains investigated in this study: 6 different RFLP subgroups AceF-A, -B, -C, -D, -E and –F. We confirmed that strains belonging to 4 subgroups, AceF-A, -B, -C and -E were present in north-east Italy, where a large number of the samples were processed. The strains of AceF-A and -E subgroups were the predominant ones (21.6% and 17.0%, respectively) and mixed infections of AceF-A+E subgroups (17.0%), and AceF-B+E (14.8%) subgroups were quite common.

Keywords: phytoplasma, European stone fruit yellows, molecular differentiation, sequencing

## Introduction

'Candidatus *Phytoplasma prunorum*' is the causal agent of European stone fruit yellows (ESFY), a quarantine phytoplasma disease mainly present in Europe and also recently reported in Turkey (Sertkaya et al., 2005). European stone fruit yellows have a wide range of host plants among cultivated and wild stone fruits species, which show large differences in terms of symptom expression and susceptibility (Carraro et al., 2002; 2004). *Prunus armeniaca* (apricot) and *P. salicina* (Japanese plum) show a high susceptibility and sensitivity to the disease. 'Ca. *P. prunorum*' is specifically transmitted by the psyllid *Cacopsylla pruni* (Scopoli) (Carraro et al., 1998; 2001) and, together with 'Ca. *P. mali*' and 'Ca. *P. pyri*', belongs to a major phylogenetic group, the apple proliferation (AP) phytoplasma group (16SrX) (Seemüller and Schneider, 2004). Conventional detection of fruit tree phytoplasmas is mainly based on nested PCR using 16S rDNA universal or group specific primer pairs, followed by identification using RFLP analyses. New molecular typing tools for fruit tree phytoplasmas belonging to the 16SrX phytoplasma group have recently been developed based on the non-ribosomal genes *aceF*, *pnp*, *imp*, and *SecY* (Danet et al., 2007; 2008). In the present work we chose to perform a PCR-RFLP method based on the *aceF* gene for differentiation of 'Ca. *P. prunorum*'. This genetic marker showed high variability among strains of the 16SrX group, moreover, it allowed for the differentiation of French hypovirulent 'Ca. *P. prunorum*' strains from the virulent ones (Danet et al., 2008).

## Material and methods

Plant material and phytoplasma reference strains: Most of the stone fruit samples were collected in north-east Italy (Friuli Venezia Giulia, FVG) from different locations during the years 2007-2008. Some of Italian samples, with a geographical origin indicated as FVG, Udine, were not collected in the field and they became infected by *C. pruni* under controlled conditions (Table 2).

Samples from Turkey and Bosnia and Herzegovina (BiH) were also included in the work to explore variability (Table 2). One Azerbaijan strain (Azer 10) and some French hypovirulent (PVC-LA8-HypV, B7-HypV) and virulent (G32, Psalor, ECA-M200, ESFY 042-1, ESFY 14-1, ESFY 293-4) strains that were shown to be genetically different in a previous study (Danet et al., 2008) were used as reference strains. Phytoplasma strains maintained in periwinkle LNS2, LNp (= ESFY) and GSFY2 were also used as reference strains in this work.

Nucleic acid extraction and 'Ca. *P. prunorum*' differentiation based on a PCR/RFLP method using the *aceF* gene: Total DNA from periwinkle-maintained phytoplasma reference strains was extracted using the CTAB extraction method (Doyle and Doyle, 1990). Plant total DNA was extracted from stone fruit leaf mid-veins according to a previously recorded protocol (Doyle and Doyle, 1990) that was slightly modified. The presence of phytoplasmas in plant samples was determined by the conventional nested-PCR procedure based on 16S rDNA using P1/P7 or P1/16S-SR primer pairs (Lee et al., 2004) in direct PCR followed by fO1/rO1 primers (Lorenz et al., 1995) in nested PCR. Restriction fragment length polymorphism (RFLP) analyses of fO1/rO1 PCR products were performed with *Ssp*I and *Rsa*I enzymes in order to identify 'Ca. *P. prunorum*' positive samples.

All of the positive samples obtained using the first method were then analysed with the non-ribosomal method based on the *aceF* gene. The *aceF* gene was amplified by nested PCR using the recently published primers AceFf1/AceFr1 followed by AceFf2/AceFr2 (Danet et al., 2008). The amplification protocol was slightly modified from the previously published protocol by Danet *et al.* (2008). Direct and nested PCR were performed as follows: initial denaturation at 94 °C for 2 min, followed by 35 cycles consisting of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 45 s, and by a final extension step at 72 °C for 8 min. The first amplification products were diluted by 1:30 and dilutions were used as a template in nested PCR. Five microlitres of PCR products were visualized by electrophoresis in 1% agarose gel and stained with GelRed™ (Biotium, Inc., Hayward, CA).

Sequencing of a portion (about 500 bp) of the *aceF* gene was performed on some of the positive samples and the nested-PCR products were purified using a Wizard® SV Gel and the PCR Clean-Up System Kit (Promega, WI, USA). Sequencing was performed with an automated DNA sequencer (ABI Prism Model 3730, Applied Biosystems, CA, USA) at the Genelab (ENEA Casaccia, Rome, Italy) using the forward primer. The obtained a*ceF* gene sequences were aligned using BioEdit v7.0.0 software package (Hall, 1999) and visually inspected.

Based on the alignment, a few restriction enzymes were selected for 'Ca. *P. prunorum*' strain differentiation. RFLP analyses were carried out with *Bpi*I, *Hae*III (Fermentas, Lithuania) and *Tsp*509I (New England BioLabs, USA) enzymes as recommended by the manufacturer to cleave AceFf2/AceFr2 nested-PCR products obtained from the phytoplasma reference strains and all positive field collected samples. The digested products were then separated by electrophoresis through a 10% polyacrylamide gel in 1X TBE (*Tsp*509I digested products) or a 2-3% MS-6 Metagel Agarose (Conda) in 1X TBE (*Bpi*I and *Hae*III digested products).

## Results

All of the phytoplasma strains from stone fruit samples showing identical 16S rDNA-based RFLP profiles to 'Ca. *P. prunorum*' reference strains (16SrX-B) were selected for further characterization. Nested-PCR products 797 bp long were obtained using *aceF* gene primers from all selected samples from Italy (FVG) (88 samples), Turkey (6 samples) and BiH (5 samples), and from all used reference strains.

Analysis of the obtained partial *aceF* gene sequences (about 500 bp) enabled four point mutations altering endonuclease restriction sites to be distinguished. The endonucleases whose restriction sites were deleted or created by single base substitutions were *Bpi*I, *Hae*III and *Tsp*509I, and these were used in RFLP analyses of the *aceF* gene sequences for 'Ca. *P. prunorum*' strain differentiation.

The putative restriction sites of *Bbs*I (*Bpi*I), *Hae*III and *Tsp*509I on the *aceF* gene sequences of some representative 'Ca. *P. prunorum*' strains are shown in Figure 1. Actual RFLP pattern types of *Bpi*I, *Hae*III and *Tsp*509I enzymes useful for strain differentiation are illustrated in Figure 2. *Bpi*I and *Hae*III enzymes generated two different profiles, one profile was undigested and the second one constituted by two different fragments. *Tsp*509I enzyme enabled three different pattern types to be distinguished. As shown in Table 1, combining the pattern types obtained with the three restriction enzymes, it was possible to distinguish between the 'Ca. *P. prunorum*' strains investigated in this study: 6 different RFLP subgroups AceF-A, -B, -C, -D, -E and -F.

**Tab. 1** Patterns produced by RFLP analyses of *aceF* gene sequences from representative strains of 'Ca. *P. prunorum*'.

| | | RFLP pattern type with restriction enzyme | | | |
|---|---|---|---|---|---|
| **Phytoplasma strain** | **Origin** | ***Bpi*I** | ***Hae*III** | ***Tsp*509I** | **AceF-subgroup** |
| Apricot SP5-36 | Italy | 1 | 1 | 1 | A |
| LNS2 - *C. roseus* | Italy | 1 | 1 | 2 | B |
| Peach 31 | BiH | 1 | 2 | 1 | C |
| Psalor | France | 1 | 1 | 3 | D |
| LNp - *C. roseus* | Italy | 2 | 1 | 2 | E |
| Azer 10 | Azerbaijan | 2 | 2 | 2 | F |

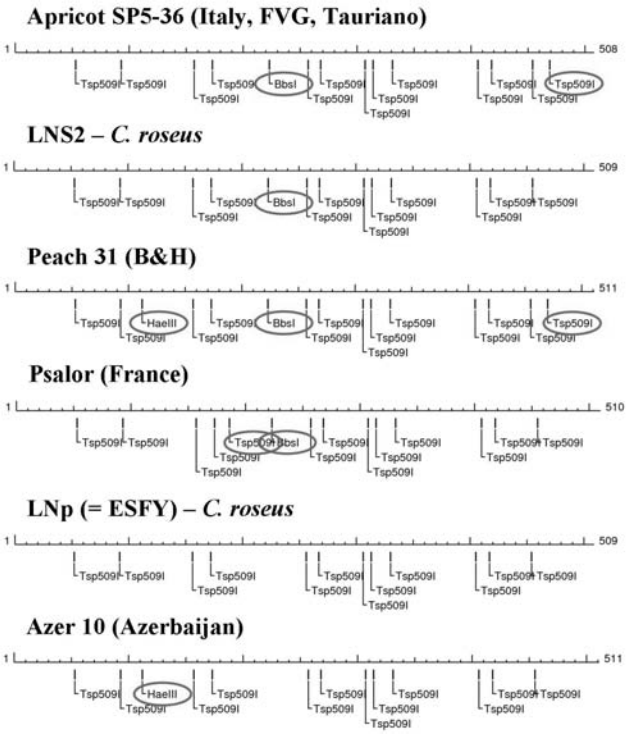


**Fig. 1** Putative restriction sites of *Bbs*I (*Bpi*I), *Hae*III and *Tsp*509I enzymes in partial *aceF* gene sequences amplified by nested PCR with primer pair AceFf1/AceFr1 followed by AceFf2/AceFr2 from representative 'Ca. *P. prunorum*' strains.

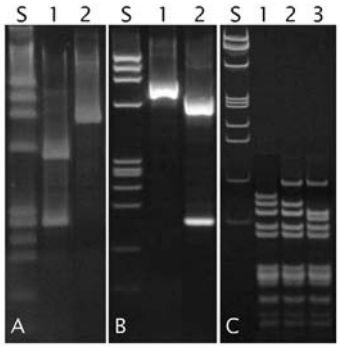


**Fig. 2** Actual RFLP pattern types of AceFf2/AceFr2 nested-PCR products digested with restriction enzymes (A) *Bpi*I, (B) *Hae*III and (C) *Tsp*509I. 1, 2, 3: RFLP pattern types. S: Φ174 *Hae*III digested (New England BioLabs, USA).

All of the results obtained by RFLP analyses on field samples from Italy, Turkey and BiH are summarized in Table 2. In north-east Italy, 4 different 'Ca. *P. prunorum*' strains were found to be present belonging to AceF-A, -B, -C, -E subgroups. The 'Ca. *P. prunorum*' strains mostly found in north-east Italy were those belonging to AceF-A (19/88; 21.6%) and -E (15/88; 17.0%) subgroups. Mixed RFLP patterns were quite common, in fact half (44/88; 50.0%) of the analysed samples showed overlapping profiles, especially with *Bpi*I and *Tsp*509I. These results indicated that mixed infections were quite a widespread phenomenon in the orchards that were inspected in north-east Italy. Since a single plant sample could show mixed profiles with more than one enzyme, in order to simplify the interpretation of the RFLP results it was hypothesized that mixed infections are derived from no more than two different strains present at the same time within the plants. The most frequent mixed infections were represented by strains belonging to AceF-A+E subgroups (15/88, 17.0%) and AceF-B+E (13/88, 14.8%) subgroups. Among the few plant samples analysed from Turkey and BiH, 'Ca. *P. prunorum*' strains belonging to the AceF-C subgroup seemed to be as important as the strains of the AceF-A subgroup (Table 2).

**Tab. 2** Results obtained by RFLP analyses of *aceF* gene sequences from stone fruits infected with 'Ca. *P. prunorum*' strains and phytoplasma reference strains (in bold) from France, Azerbaijan and those maintained in periwinkles.

| **Geographical origin** | **Infected host/Reference strain** | | **AceF-subgroup no. of samples/tested samples** |
|---|---|---|---|
| Italy (FVG, Tauriano) | Apricot | | A (4/14), B (1/14), E(1/14), A+B (1/14), A+C (1/14), A+E (3/14), B+E (2/14), C+E (1/14) |
| Italy (FVG, Gaio) | Apricot | | A (7/16), B (2/16), C (2/16), E (4/16), B+E (1/16) |
| Italy (FVG, Galleriano) | Apricot | | A (6/39), B (1/39), C (2/39), E (5/39), A+B (2/39), A+C (5/39), A+E (7/39), B+E (8/39), C+E (3/39) |
| Italy (FVG, Udine)* | Japanese plum, apricot, *Prunus mahaleb*, *P. cerasifera*, *P. tomentosa* | | A (2/19), B (1/19), C (1/19), E (5/19), A+E (5/19), B+E (2/19), C+E (3/19) |
| Turkey | plum, apricot, almond, peach | | A (2/6), C (2/6), A+C (2/6) |
| BiH | apricot, peach | | A (3/5), C (2/5) |
| France | *Prunus* sp. | G32 | B |
| | | Psalor | D |
| | | ECA-M200 | B |
| | | ESFY 042-1 | B |
| | | ESFY 14-1 | B |
| | | ESFY 293-4 | B |
| | | PVC-LA8-HypV** | A |
| | | B7-HypV** | A |
| Azerbaijan | *Prunus* sp. - Azer 10 | | F |
| Italy | *C. roseus* - LNp (= ESFY) | | E |
| Germany | *C. roseus* - GSFY2 | | B |
| Italy | *C. roseus* - LNS2 | | B |

* Stone fruit trees maintained in controlled conditions under a screenhouse, and exposed to infection by *C. pruni*. ** French 'Ca. *P. prunorum*' hypovirulent strains

Among the phytoplasma reference strains from France, the Psalor strain represented a different subgroup indicated by AceF-D, and the reference strain Azer 10 from Azerbaijan represented another different subgroup indicated by AceF-F (Tables 1 and 2). Using the *Bpi*I enzyme it was possible to differentiate Italian LNp reference strains from the other '*Ca*. P. prunorum' reference strains maintained in the periwinkles.

## Discussion

RFLP analyses and sequencing of the 16S rRNA gene and a non-ribosomal gene did not enable differentiation between 'Ca. *P. prunorum*' strains (Jarausch et al., 2000). Recently, genomic variability between 'Ca. *P. prunorum*' strains was shown using a molecular approach based on a multi-locus sequence typing (MLST) strategy (Danet et al., 2007; 2008). One of the four non-ribosomal genetic loci used in the MLST was the *aceF* gene, which was chosen to develop a PCR-RFLP method for strain differentiation because from preliminary results it seemed possible to distinguish hypovirulent strains from virulent ones using this gene (Danet et al., 2008). The PCR-RFLP method based on the *aceF* gene described in this work confirmed the genetic variability among 'Ca. *P. prunorum*' (16SrX-B) strains and distinguished 6 different RFLP AceF-subgroups among the analysed strains.

The results obtained by the RFLP analysis showed that in north-east Italy (FVG), where a large number of samples were processed, it was possible to find a high variability among the strains tested, since four different subgroups were present. It also demonstrated that the strains belonging to AceF-A and -E subgroups were the predominant ones and that mixed infection by the two strains was also quite common together with the mixed infection by strains of AceF-B and -E subgroups. From the results obtained from analysing the samples collected in the screenhouse it appeared that *C. pruni* is able to transmit all of the 'Ca. *P. prunorum*' strains present in north-east Italy. The high percentage of mixed infections could be explained by the presence of several strains in the surveyed locations, high vector population densities and by recurring phytoplama inoculations by the vector year after year.

The French hypovirulent strains were characterized as belonging to the subgroup AceF-A, which is quite a widespread subgroup in north-east Italy and has also been shown to be present in Turkey and BiH. In many cases, this particular type of 'Ca. *P. prunorum*' strain was associated with plants exhibiting clear symptoms of European stone fruit yellows in Italy (FVG), Turkey and BiH. For this reason we cannot confirm that this molecular marker allowed for differentiation between hypovirulent and virulent strains, as it appeared to do in the work by Danet *et al.* (2008). The French strain Psalor and the Azerbaijan strain Azer 10 were found to be molecularly different from all of the other analysed strains, confirming previous published data by Danet et al. (2008). In particular, the strain Azer 10 was shown to be genetically divergent, exhibiting 10 nucleotide substitutions when compared to the reference strain GSFY2.

The molecular method described in this work represents a valid tool in epidemiological studies devoted to elucidate the relationships between plant host/phytoplasma vector. This work can be considered as the first step towards future studies that will be focused on the characterization of 'Ca. *P. prunorum*' strains present in the vectors, and on the biological properties of different strains, such as transmissibility by vectors and virulence.

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