Molecular characterization of 'Candidatus *Phytoplasma prunorum*' in *Cacopsilla pruni* insect vector

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Abstract

Recent investigations on molecular characterization of the 'Candidatus *Phytoplasma prunorum*' (16SrX-B subgroup), causal agent of the European Stone Fruit Yellows (ESFY) syndrome, on the non ribosomal *tuf* gene resulted in the finding of two groups of isolates, named 'type a' and 'type b', both with a distinctive geographical distribution in Italian stone fruit growing areas (Ferretti et al., 2007 and 2008). Considering the role of *Cacopsylla pruni* (Scopoli) in the epidemiological cycle of the disease, the presence of the two groups of isolates has also been investigated in infected psyllid individuals from different Italian areas. Both types have been identified in *C. pruni* specimens collected on apricot, plum and wild *Prunus* species, confirming the geographical distribution and the percentages of spread of the two isolates.

Keywords: ESFY, phytoplasma, characterization, *tuf* gene, insect vector

Introduction

'Candidatus *Phytoplasma prunorum'* (16Sr X-B subgroup), the causal agent of the European Stone Fruit Yellows (ESFY) syndrome, is spreading in all Italian stone fruit growing areas. Leaf rolling and discoloration on single branches develop rapidly into leaf chlorosis. Sometimes, necrosis and dieback are observed in the same season. The Psyllid *Cacopsylla pruni* is the specific vector of the phytoplasma. This insect species is characterised by the presence of one generation per year: adults, overwintering on coniferous and other forest plants, migrate at the beginning of spring on the primary host, represented by wild and cultivated *Prunus* spp., where they lay their eggs.

Recently, a molecular investigation revealed the presence of two different groups of isolates of 'Ca. *P. prunorum*', reported as 'type a' and 'type b', on the basis of the molecular variability of the non ribosomal *tuf* gene (Ferretti et al., 2007). Further investigation, carried out on a large number of plant samples coming from infected orchards located in several Italian regions, confirmed the presence of these isolates and their well defined geographical distribution (Ferretti et al., 2008).

In order to also verify this molecular variability in the phytoplasma harboured in the insect vector, individuals of *C. pruni* were collected in several Northern Italian regions, where symptoms resembling the ESFY disease were frequently observed, during the course of recent years, in many stone fruit orchards. The surveys were carried out in orchards in which the distribution of the two isolates has been already defined.

In this paper, the molecular characterization of 'Ca. *P. prunorum*' found in insect samples is reported. The interdependence of isolates identified in insect and plant samples coming from the same growing areas was investigated.

Materials and methods

Individuals of *C. pruni* were captured by means of yellow sticky traps and sweep-net from April to May in ESFY-affected orchards located in several Northern Italian regions (namely Lombardia, Trentino, Veneto and Emilia Romagna), which had been previously surveyed for the detection and characterization of 'Ca. *P. prunorum*'. Insects were collected both from cultivated apricot and peach varieties and wild *Prunus* species (*P. cerasifera* and *P. spinosa*) growing alongside the investigated orchards.

Total DNA was extracted from single specimens according to the procedures described by Marzachì et al., (1998) and submitted to PCR amplification of 16S gene fragments, using the primer pairs P1/P7 (Schneider et al., 1995; Deng and Hiruki, 1991), and fO1/rO1(Lorenz et al., 1995) in direct and nested PCR, respectively. Total DNA from 72 positive individuals was then analyzed by means of specific amplification of non-ribosomal DNA fragments of the phytoplasma *tuf* gene. The primer pairs tuf1f/1r and tuf2f/2r, specifically designed on the *tuf* gene of the 16SrX phytoplasma group, were used in direct and nested PCR, respectively. Amplification was performed as follows: 3 min at 94 °C, 40 cycles of 1 min at 93°C (denaturation), 1 min at 45°C (annealing), 1 min and 30 sec at 72 °C (extension) and a final extension of 15 min at 72°C. Total DNA from 16SrX-A, 16SrX-B and 16SrX-C phytoplasma infected plants and from an healthy apricot were used as controls.

The obtained amplicons were submitted to the RFLP (Restriction Fragments Length Polymorphism) analysis, following digestion with the *Nla*III restriction enzyme. The obtained profiles were analysed after separation by electrophoresis on 5% polyacrilamide gel stained in ethidium bromide and successive visualization under UV light.

Results

Amplification products of the expected size (1120 bp) were obtained in nested PCR with the internal primer pair tuf2f/2r from 66 out of 72 (91.6%) individuals which had previously tested positive by 16S gene molecular analysis (Fig. 1). In many cases, amplicons of the right size were also obtained through direct PCR.

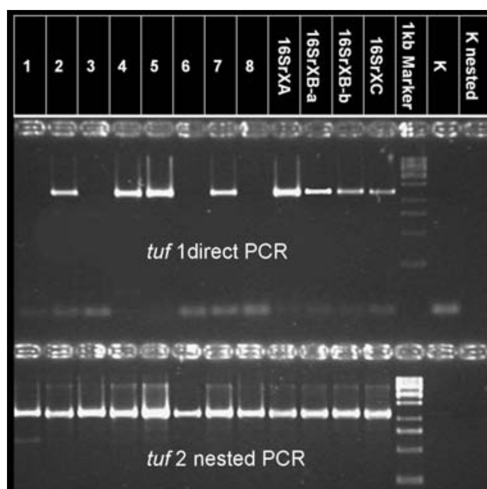

Fig. 1 Agarose gel of direct and nested-PCR on *tuf* gene. Lanes 1-8: insect samples; lanes 9-12 reference strains (16SrX-A, 16SrX-B 'tuf type a', 16SrX-B 'tuf type b', 16 SrX-C); lane 13: 1 kb DNA marker; lanes 14-15: water controls.

RFLP analysis performed on the *tuf* gene amplified fragments showed the presence of two distinct restriction profiles, referable to 'tuf type a' and 'b' (Fig. 2). No mixed infections by either tuf types in single individuals were ever detected in the tested insects (Tab. 1).

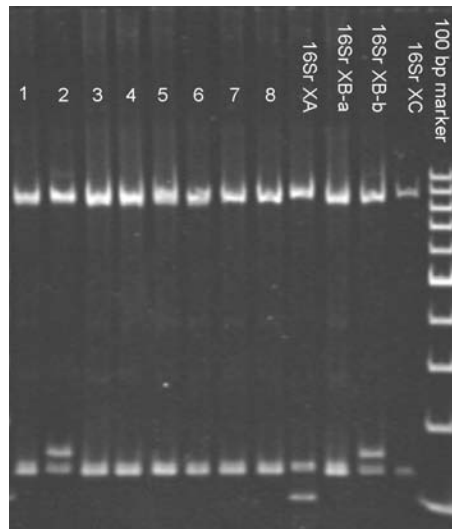

Fig. 2 RFLP analysis of the *tuf* gene amplicons after digestion with *Nla*III restriction enzyme. Lane 1-8: insect samples; lanes 9-12 reference strains (16SrX-A, 16SrX-B 'tuf type a', 16SrX-B 'tuf type b', 16 SrX-C); lane 13: 100 bp DNA marker

Tab. 1 Percentages of distribution of 'tuf type a' and 'b' isolates identified in *C. pruni* individuals collected in four different Italian regions.

Region	'tuf type' distribution (*) (%)	N° orchards	*C. pruni* individuals captured in the investigated orchard: Host plant	N° positive insects (positive/total)	'tuf type' 'a'	'tuf type' 'b'
Trentino	a (77.3%); b (22,7%)	5	Apricot: *P. cerasifera*; *P. spinosa*	10/13	8 (80%)	2 (20%)
Friuli-V.G.	a (94,4%); b (5,6%)	1	Apricot	5/6	5 (100%)	-
Lombardia	a (92,3%); b (7,7%)	3	*P. spinosa*	26/26	24 (92,3%)	2 (7,7%)
Emilia-R.	a (100%)	3	*P. spinosa*	25/27	25 (100%)	-
Totale	-	12	-	66/72	62 (96.8%)	4 (3,2%)

(*) Data previously published (Ferretti et al., 2008)

In the 25 *C. pruni* specimen coming from 3 orchards located in Emilia-Romagna and in the 5 insect samples coming from one orchard of the Friuli V.G. region, only the 'tuf type a' was found, whereas on a total of 36 insect individuals from 8 orchards located in Trentino and Lombardia, both tuf types were identified (Tab. 1). In these last two regions, an infection rate of *C. pruni* by 'tuf type a' of 80.0 % and 92.3 % respectively was detected.

Discussion

The primer pairs tuf1f/1r and tuf2f/2r were confirmed to be specific and suitable for the characterization of 'Ca. *P. prunorum*' in insect samples and they also resulted useful for the phytoplasma detection. In fact, the percentage of positive insect specimens was very similar to that obtained in the 16S gene molecular analysis. The PCR/RFLP-based method using the *tuf* gene confirmed the differentiation of the 'Ca. *P. prunorum*' in two distinct types. The same result was observed for phytoplasma harboured from the *C. pruni* specimen, as previously identified in ESFY-affected plants (Ferretti et al., 2007).

Previous studies on the geographical distribution of the two 'tuf types' on infected plants revealed the presence of the only 'type a' in the Emilia-Romagna region, whereas in Friuli V.G., Trentino and Lombardia, both types have been found inside the infected orchards (Ferretti et al., 2008). This geographical distribution of types was also confirmed in the insect specimens, with the exception of the samples coming from the Friuli-V.G. region, where both types were identified on *Prunus* plant species, but only the 'type a' was found in *C. pruni* specimens. Nevertheless, the quality of

the data could have been influenced by the low insect population density recorded during the sampling season, and the subsequent small number of tested insects.

The presence of two distinct isolates of ‘Ca. *P. prunorum*’, on the basis of the molecular variability of the non ribosomal *tuf* gene, was confirmed at different stages of the phytoplasma epidemiological cycle; the correspondence between the ‘tuf types’, identified in plants and insect vectors from the same infected areas, enhances the relevance of this molecular characterization as an effective tool for epidemiological investigations.

Acknowledgements

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