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Imp and *sec*Y, two new markers for MLST (multilocus sequence typing) in the 16SrX phytoplasma taxonomic group

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Abstract

To investigate the genetic variability of fruit tree phytoplasmas belonging to the 16SrX ribosomal group, we used a multilocus sequence typing strategy (MLST). Sequences of four non-ribosomal genetic loci (*aceF*, *pnp, imp* and *secY*) were determined among a collection of '*Candidatus* Phytoplasma prunorum', '*Ca.* P. mali' and '*Ca.* P. pyri' isolates. Sequences alignment and phylogenetic analyses confirmed the classification based on16S rDNA phylogeny. The four genetic *loci* displayed specific signatures clearly correlated with the recent definition of the corresponding '*Candidatus* species'. However, a total congruence was not observed inside '*Ca*. species', between phylogenetic trees constructed with the different *loci.* A divergent '*Ca*. P. prunorum' variant from Azerbaijan, recently characterized through *ace*F sequencing, presented a divergent *imp* gene. The previously characterized '*Ca*. P. prunorum' hypovirulent strains had the same *aceF* sequence but were discriminated with the *imp* marker. '*Ca*. P. pyri 'strains detected in Lebanese pear decline isolates also form a distinct cluster according to *aceF* and *imp* markers*. Imp* and *secY* phylogenetic analyses pointed out a new '*Ca.* P. prunorum' cluster comprising strains never detected in psyllid vectors. '*Ca*. P. prunorum' strains were distributed among all phylogenetic group identified. For '*Ca*. P.mali', discrimination of apple proliferation isolates was in agreement with previous studies.

Key words: *Mollicutes*, variability, fruit tree diseases, pear decline, apple proliferation, European stone fruit yellows, *ace*F, *pnp*, phylogenetic analyses.

Introduction

The major phytoplasmas affecting fruit trees are '*Candidatus* Phytoplasma prunorum' (European stone fruit yellows), '*Ca.* P. mali' (apple proliferation) and '*Ca.* P. pyri' responsible for the pear decline. They belong to the ribosomal group 16SrX, characterized by a high 16SrDNA homology (>98 %) (Seemüller *et al.*, 2004). Variation in virulence has been reported among '*Ca.* P. prunorum' isolates (Kison *et al.*, 2001) as well as among '*Ca.* P. pyri' isolates, but these isolates could not be distinguished on molecular bases. In order to investigate genetic variability of these fruit tree phytoplasmas, two molecular typing tools based on *aceF* and *pnp,* for a MLST strategy were developed (Danet *et al*., 2007). To obtain a more accurate genotyping of 16SrX phytoplasma strains, two new markers *imp* and *sec*Y were employed. In the present work their sequence variability in a large panel of 16SrX phytoplasmas is reported.

Materials and methods

Samples from diseased *Prunus* (74 isolates), *Malus* (16 isolates) or *Pyrus* trees (14 isolates) were collected from different regions in Azerbaijan, Austria, Croatia, France, Germany, Italy, Lebanon, Romania, Spain, Switzerland and United-Kingdom. Reference strains: GSFY2, ESFY, AP15, AT, and PD1, were propagated in periwinkle by graft inoculation and used as positive controls. Psyllids *Cacopsylla pruni* were collected in the south of France. DNA extraction was performed from plants and insects samples according to a published CTAB procedure (Maixner *et al*., 1995).

To amplify the *imp* locus, specific primers according to published sequence data, were designed (Morton *et al*., 2003). To amplify *sec*Y *locus* of the fruit three phytoplasmas, polyvalent degenerated primers were used, chosen in conserved regions of *secY* gene, as defined through the comparison of *secY* sequences of '*Ca*. P. asteris', FD phytoplasma, stolbur phytoplasma (unpublished data) and '*Ca.* P. mali' (kindly provided by B. Schneider). *Imp* and *sec*Y *loci* were amplified by nested PCR (cycle, and conditions, unpublished data). All PCR products were sequenced using the nested PCR primers on ABI-PRISM. Sequences were aligned and compared using the ClustalW program. Phylogenetic analyses were performed by the maximum of parsimony method using MEGA3.1 and tree branching consistency evaluated by bootstrapping.

Results

The *imp* marker displayed an important variability with a mutation rate reaching 36% between '*Ca.* P. prunorum' and '*Ca.* P. mali' isolates, 29% between '*Ca.* P. prunorum' and '*Ca.* P. pyri' isolates, and 28% between '*Ca.* P. mali' and '*Ca.* P. pyri' isolates. As a comparison, 11%, 12% and 10% was found for *ace*F, 7%, 6%

and 5% for *pnp* and 8%, 7% and 10% for *sec*Y marker. Sequence comparisons based on *ace*F and *imp* sequences permitted to distinguish a geographic variant of '*Ca*. P. prunorum' from Azerbaijan with 9 and 16 nucleotide substitutions respectively. Lebanese '*Ca*. P. pyri' variants presented respectively 3 and 25 substitutions for these genes (figure 1).

Results of phylogenetic analyses of *ace*F sequences previously clustered hypovirulent strains of '*Ca*. P. prunorum' in a monophyletic group (Danet *et al*., 2007). It was not the case when analysing *imp* gene sequences (figure 1). The described distinction between '*Ca.* P. mali' strains AP type and '*Ca.* P. mali' strains AT type (Jarausch *et al.*, 1994), is confirmed with *imp* and *sec*Y diversity. 45 nucleotides for *imp* and 14 nucleotides for *sec*Y were different in sequence from AP and AT type strains. *Sec*Y and *imp* marker phylogenetic analyses clustered some '*Ca*. P. prunorum' strains suspected to be non-circulative, in a monophyletic group from a minimum 1 to a maximum of 8 divergent nucleotides by comparison to the others 'Ca. P. prunorum' strains *sec*Y and *imp* sequences.

Finally, whereas the *aceF* marker permitted to discriminate 7 strains into '*Ca*. P. prunorum', 2 strains into '*Ca*. P. pyri' and 4 strains into '*Ca*. P. mali', *pnp*, *imp* and *sec*Y allowed to discriminate 10, 9, 3 strains of '*Ca*. P. prunorum'; 5, 8 and 1 strains of '*Ca*. P. pyri'; 5, 5 and 2 strains of '*Ca*. P. mali'.

Discussion

The description of the genetic variability of 16SrX phytoplasmas should be improved by the use of *imp* and *sec*Y sequence typing. We underscored for the first time the *imp* and *sec*Y genetic variability of 16SrX phytoplasma strains.

We did not observe a total congruence between phylogenetic reconstructions for all the genes analyzed. This is certainly in agreement with the differences of the gene functions. Indeed, whereas *ace*F and *pnp* loci respectively encode a component of pyruvate dehydrogenase and the polynucleotide phosphorylase, *i.e.* two components of cytoplasmic metabolic pathways, the *imp* and *sec*Y genes respectively encode a membrane surface protein and a component of the protein secretion machinery. However, the lack of total congruence might be interesting as it may increase the discriminating power of MLST. In conclusion, amplification and sequencing of *imp* and *sec*Y genes increase the list of the typing tools for epidemiology and will allow a more precise documentation of the variability in this group of phytoplasmas.

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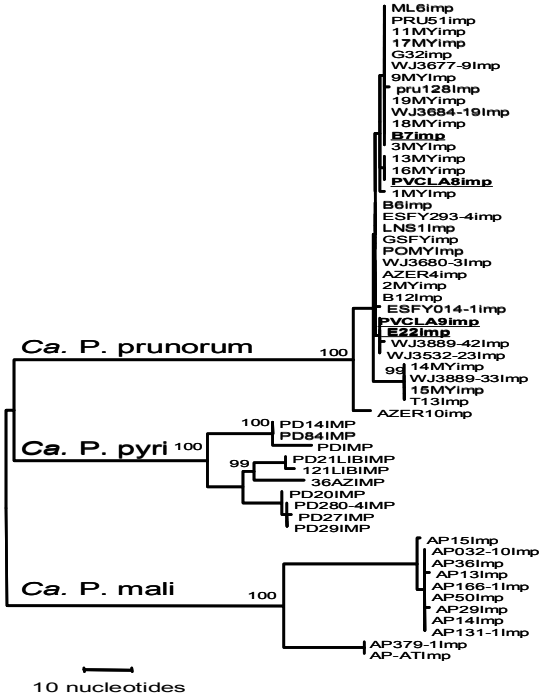

Figure 1. Maximum parsimony analysis of *imp* sequences. Hypovirulent strains are underlined.

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