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Deep amplicon sequencing reveals mixed phytoplasma infection within single grapevine plants

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Abstract

The diversity of phytoplasmas within single plants has not yet been fully investigated. In this project, deep amplicon sequencing was used to generate 50,926 phytoplasma sequences from 11 phytoplasma-infected grapevine samples from a PCR amplicon in the 5' end of the 16S region. After clustering and alignment to phytoplasma reference sequences it was shown that the phytoplasmas in the 11 plants belonged to diverse 16Sr groups and that a high number of single nucleotide polymorphisms were present.

Key words: pyrosequencing, phytoplasma, population, molecular identification.

Introduction

Phytoplasmas within single plants can be considered as populations of individuals, and as phytoplasmas are obligate parasites of plants, there are no possibilities to know unless after obtaining single clones. For identification of phytoplasmas, PCR is usually followed by RFLP or by sequencing the PCR product directly or by sequencing cloned PCR products. This procedure will, however, not show the diversity of the population, as only the most frequent genotypes will be detected and identified, unless many clones are sequenced.

Deep amplicon sequencing has been used to analyse complex microbial communities in a number of environments (Huse *et al*., 2008), but never applied to phytoplasma detection. In this project, we selected a number of grapevine samples (figure 1), in which mixed phytoplasma infections was preliminary detected by nested-PCR technique, for deep amplicon sequencing on the Roche Genome Sequencer FLX system preliminary experiments.

Materials and methods

Samples employed were selected form those that are routinely processed every year during surveys for phytoplasma detection and identification in Northern Italy 'flavescence dorée' infected areas (Veneto region). Total DNA of 30 samples was extracted from 1 g of mid-vein leaf tissue following the procedure of Prince *et al*. (1993) and 10 more samples were extracted following the procedure of Angelini *et al*., (2001). Phytoplasma detection was carried out by direct PCR on ribosomal gene and spacer region (Martini *et al*., 2002) followed by nested amplification with R16(I)F1/R1 (Lee *et al*., 1994) and $16R_{758F}/V_{1730}$ (Martini *et al*., 1999) primer pairs. RFLP analyses with *Tru*I on the first amplicons and *Taq*I on the second one allow identification of 16SrXII (stolbur) and 16SrVC/D ('flavescence dorée') phytoplasmas after 5% polyacrilamide gel electrophoresis.

Among samples showing mixed phytoplasma infection 11 were chosen for deep amplicon sequencing. Tagged primers (forward primer: primer A – TAG – 16F2n; reverse primer: primer B – ACTTAYTAAACC GCCTACR-CACC) were used for generating pyrosequencing samples. PCR cycles were: 94°C for 3 minutes followed by 35 cycles at 94°C for 15 seconds, 64°C for 30 seconds, and 72°C for 1 minute. PCR products were pooled in equimolar amounts, run on an agarose gel and a band of the correct size was excised from the gel and purified using QIAquick gel extaction kit from QIAGEN. This pool of 11 samples was sequenced on a GS FLX plate at Eurofins MWG.

Tag-sorted sequences were quality filtered using CLOTU software at the Bioportal webportal (http://www.bioportal.uio.no/). To minimize sequencing errors, only the first ~200 nucleotides of each sequence were used. Accepted sequences were clustered using CD-HIT with a 99% similarity threshold, and singleton sequences were discarded. To identify sequences, these were aligned together with reference sequences from GenBank using MEGA and phylogenetic trees were constructed.

Results

A total of 74,817 sequences were generated from the 11 field collected samples of phytoplasma infected grapevine. After quality filtering, 50,926 sequences remained, varying from 1,914 to 9,868 sequences per sample among the 11 samples. After clustering at 99% similarity threshold, sequences were aligned to a reference set of sequences of known identity. This showed that 11,730 sequences belonged to phytoplasma 16SrV group, 38,456 sequences were belonging to 16SrXII group, 576 sequences were related to '*Candidatus* Phytoplasma prunorum' whereas the last 164 sequences could not be assigned to a single phytoplasmas group.

Figure 1. Grapevine cv Chardonnay showing symptoms of phytoplasma infection.
(In colour at www.bulletinofinsectology.org)

There was a large variation in the number of sequences within each sample: 16SrV group ranged between 33 to 3,009 sequences; 16SrXII from 0 to 6,823 sequences; '*Ca*. P. prunorum' from 0 to 278 sequences. Apart for the overall grouping of phytoplasmas, a high number of single nucleotide polymorphisms were detected in the sequences.

Discussion

Phytoplasmas are unable to survive on an artificial growth media available so far, therefore identification to 16Sr group or '*Candidatus* species' must be carried out on infected plant material. This means that identification is carried out on populations of phytoplasmas rather than on individual clones, which is the case for most microorganisms. To investigate the variation of phytoplasma sequences within single plants, a number of samples were selected that showed mixed phytoplasma presence in nested-PCR, however the detection system employed was only target to the detection of 16SrV-C/D and 16SrXII phytoplasmas since these are know to be associated with the majority of the phytoplasma diseases in grapevine in Europe (Botti and Bertaccini, 2007).

These samples were subjected to deep amplicon sequencing in which the sequence of individual PCR products is determined without the need of cloning.

From the 11 samples examined a total of 50,926 good quality sequences were analysed. Several 16Sr groups/ '*Candidatus*' species were identified, but also a high number of single nucleotide polymorphisms was found. This experiment demonstrates that phytoplasmas in individual grapevine plants are composed of highly diverse populations of individuals and that there are possibility to employ pyrosequencing techniques to verify presence of mixed phytoplasmas populations in naturally infected samples.

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