

Phylogenetic analysis of phytoplasmas based on sequences derived from the *secA* and 23S rDNA genes

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Abstract

Phylogenetic analysis of a 500 bp fragment of the *secA* gene has been performed on 35 phytoplasma isolates representing 10 of the 16S rDNA groups. The lineages obtained coincide with those delineated by 16S rDNA phylogenetic analysis but much greater genetic variability was evident. In addition, phylogenetic analysis based on part of the 23S rDNA gene combined with T-RFLP (terminal restriction fragment length polymorphism) analysis of this region supports the lineages. The results clearly indicate a significant delineation between the 16SrI and 16SrXII phytoplasmas and those in other 16Sr groups, and support the proposals that the coconut lethal yellowing type phytoplasmas should be divided into three 'Candidatae species'.

Key words: Phytoplasma, phylogenetic analysis, *secA*, T-RFLP, 23S rDNA.

Introduction

To date, most phytoplasma taxonomy has been based upon the 16S rDNA gene which is highly conserved throughout phytoplasma groups. In 2004, the IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma taxonomy group proposed to accommodate phytoplasmas within the genus 'Candidatus Phytoplasma' gen. nov. in which the formal descriptions and species composition fit closely to the 16S groups of Lee *et al.* (1998) (IRPCM, 2004; Firrao *et al.*, 2005). However, it is of interest to determine how a group system based on 16S rDNA similarity compares when constructed with other genes such as the 23S rDNA or less well conserved genes such as *secA*. Whilst there are more than 200 sequences available in databases of phytoplasma 16S rDNA genes and spacer regions, there are very few sequences available for phytoplasma 23S rDNA or other genes, so comparisons of these have not been possible. Lee *et al.* (2006) studied the *secY* gene as a tool to provide finer differentiation of the 16S rDNA group I (aster yellows) phytoplasmas and this revealed 10 sub-groups within the 20 samples analysed, compared to the 6 subgroups when the same samples were analysed with 16S rDNA. Other genes have also been tested, but mainly on 16SrI group phytoplasmas where it has been relatively easy to design primers that amplify from infected plant DNA based on the complete sequences of onion yellows and aster yellows witches' broom that are now available. In this work we have developed PCR primers that amplify parts of the *secA* gene and the 23S rDNA gene from as many 16Sr groups as possible to ascertain the relatedness of the groups, with the potential that these genes might be less conserved than the 16S rDNA leading to a greater understanding of the evolutionary relatedness of phytoplasmas.

Materials and methods

Primers to amplify a 500 bp segment of the *secA* gene were designed by comparison of sequences available on PubMed for aster yellows witches' broom and onion yellows and that of lethal yellows phytoplasma (16SrIV). PCR amplification was performed in 25 µl reaction mixture using "Ready To Go PCR Beads™" (Amersham Pharmacia Biotech, Amersham, UK) containing 15 ng of template DNA and 100 ng of each primer in a MJ Research PTC200 thermocycler. PCR products were purified using Qiagen's QIAquick PCR purification kit and the cloned via Promega's pGEM T-Easy Vector System. Clones were then sequenced using a Beckman CEQ 8000 automated sequencer, and sequences were edited and aligned using CLUSTAL version X. Genetic distances were calculated using DNA neighbour-joining from the PHYLIP package and tree construction and evaluation of statistical confidence was determined by bootstrap (100 pseudoreplicates) (Felsenstein, 1985). T-RFLP was performed as described by Hodgetts *et al.* (2007).

Results

A 500 bp sequence was amplified from the *secA* gene, cloned and sequenced from 32 samples which included 6 from 16SrI, 7 from 16SrII, 4 from 16SrIII, 3 from 16SrIV, 1 from 16SrV, 3 from 16SrVI, 1 from 16SrVII, 4 from 16SrX, 1 from 16SrXI and 2 from 16SrXII ribosomal groups. A phylogenetic tree derived from the 32 samples cloned and 3 additional sequences available on-line was produced. Similarly, a 750 bp sequence that included the 16S/23S spacer region and approximately 500 bp of the 23S rDNA was cloned and sequenced from the same set of phytoplasmas, and a phylogenetic tree produced. For comparative purposes, an equivalent tree was derived from published 16S rDNA

sequences for as many of the isolates as possible. The *secA* analysis produced one subclade containing group 16SrI and 16SrXII phytoplasmas, and a second subclade containing all other phytoplasmas. When compared to the conventional 16S grouping system, a similar pattern was obtained but the 16SrI/16SrXII lineage also contained the 16SrX phytoplasmas. The *secA* analysis also revealed some significant separation of isolates within some of the 16Sr groups. In 16SrII, there was clear distinction between the 16SrII-C and 16SrII-D isolates, whilst in the 16SrIV group, there were significant differences between LY from the Americas, Ghanaian Cape St Paul Wilt, and Tanzanian LYD. Analysis of the 23S rDNA revealed similar phylogenetic relationships, and when T-RFLP was performed using this region of the genome, the technique split group 16SrI into two subgroups and similarly split group 16SrII into two separate subgroups.

Discussion

Previous analysis based primarily on the 16S rDNA gene has indicated that the aster yellows group phytoplasmas (16SrI) form a distinct subclade within the phytoplasma clade, and further analyses of this subclade using genes such as *secY* and *tuf* have subdivided this group into distinct lineages (Lee *et al.*, 2006). Phylogenetic analysis of phytoplasmas from other 16Sr groups has been more problematic because it has been difficult to find primers that can amplify genes other than 16S rDNA from these other groups. In some cases the spacer region located between the 16S and 23S rDNA genes has been used and this shows greater variation than the 16S rDNA gene, and sequence analysis of the 16S/23S spacer region for phytoplasmas results in a classification similar to that derived from 16S rDNA data, but with more detailed sub-divisions (Wang and Hiruki, 2005). In this study we have extended the analysis to cover part of the 23S rDNA along with the spacer region, and more importantly have also designed and utilised primers that can amplify a region of coding DNA (part of the *secA* gene) from all phytoplasmas tested. Analysis using these sequences has shown that the basic subclades remain the same, but the 16SrXII phytoplasmas tend to group more closely to the 16SrI phytoplasmas than to other subgroups. Similarly the analysis also indicates that there are some significant subdivisions within other subclades, particularly the 16SrII phytoplasmas which form two distinct subgroups, and the coconut lethal yellowing type phytoplasmas (16SrIV) which split into the LY phytoplasmas from the Americas, the West Af-

rican lethal yellowing-like diseases (e.g. Cape St Paul Wilt from Ghana), and the East African lethal yellowing-like diseases. These results would support the classification of the 16SrIV phytoplasmas into three distinct 'Candidate species', 'Ca. P. palmae', 'Ca. P. cocosnigeriae' and 'Ca. P. cocostanzaniae' as proposed at the X International Congress of the IOM, 1994 and reported by Firrao *et al.* (2005).

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