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Virtual RFLP analysis of 16S rDNA sequences identifies new subgroups in the clover proliferation phytoplasma group

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Abstract

Phytoplasmas are insect-transmitted, phloem-inhabiting, cell wall-less bacteria that cause numerous diseases in several hundred plant species. In adaptation to diverse plant hosts and insect vectors, phytoplasma evolution has given rise to widely divergent lineages. As phytoplasmas are unculturable and their phenotypic characters are largely inaccessible, molecular analyses of conserved gene sequences have become rational means for phytoplasma differentiation and classification. Extending our recent efforts in exploitation of computer-simulated 16S rDNA RFLP analysis and virtual gel plotting for rapid classification of phytoplasmas, we have developed an algorithm for automated RFLP pattern comparison and similarity coefficient calculation. Such streamlined virtual RFLP pattern analysis of 16S rDNA sequences has led to identification of new pattern types and potential new subgroups in the clover proliferation phytoplasma group.

Key words: phytoplasma classification, virtual RFLP analysis, similarity coefficient.

Introduction

Phytoplasmas are a large group of cell wall-less bacteria that cause diseases in several hundred plant species including economically important vegetable, cereal, fruit, and forest crops worldwide (McCoy *et al*., 1989; Lee *et al*., 2000). Phytoplasmas reside in sieve cells of plant phloem tissue and are transmitted by phloem-feeding insect vectors (Tsai, 1979). Together with acholeplasmas, mycoplasmas, spiroplasmas, and other cell wall-less bacteria, phytoplasmas are classified in class *Mollicutes*. Despite their descent from a common ancestral *Clostridium*-like low G+C gram-positive bacterium, and despite their monophyletic status within the *Mollicutes* phylogenetic tree, phytoplasmas have evolved to give rise to widely divergent lineages. Since phytoplasmas cannot be cultured in cell-free medium, their identification and classification have relied mainly on restriction fragment length polymorphism analysis of 16S rRNA and other conserved genes (Lee *et al*., 1993; 2000). A recent effort in exploitation of computer-simulated RFLP analysis facilitated considerably the implementation of 16S RNA gene sequence-based phytoplasma classification scheme and expanded the number of phytoplasma 16Sr groups to 28 (Wei *et al*., 2007). In the present study, combining *in silico* 16S rDNA RFLP analysis and automated calculation of pattern similarity coefficient, new phytoplasma subgroups in the clover proliferation group (group 16SrVI) were identified.

Materials and methods

Sequence retrieval, alignment, and cladistic analysis

Phytoplasma 16S rRNA gene sequences were retrieved online from the National Center for Biotechnology Information (NCBI)'s nucleotide sequence database at http://www.ncbi.nlm.nih.gov/gquery/gquery.fcgi. For the purpose of cladistic analysis, 16S rDNA sequences from representative taxa of non-phytoplasma mollicutes as well as Gram-positive low G+C walled bacteria were also retrieved from the nucleotide sequence database. The 16S rDNA sequences, compiled in FASTA format, were aligned using ClustalX (V1.83) program. Each aligned sequence was trimmed to an approximately 1.25-kb fragment (termed F2nR2 region hereafter) that was bounded by the two conserved nucleotide blocks corresponding to the annealing sites for phytoplasma-universal 16S rDNA primer pair R16F2n/R16R2 (Gundersen and Lee, 1996). The trimmed sequences were realigned, and the final alignment was converted to MEGA format for cladistic analyses. Maximum-parsimony analysis was conducted with the software MEGA3 using close neighbor interchange (CNI) algorithm. The initial tree for the CNI search was created by random addition for 10 replications. Analyses reliability was subjected to a bootstrap test with 100 replicates.

In silico restriction enzyme digestion and automated similarity calculation

Each trimmed sequence was exported to pDRAW32 program for *in silico* digestion with 17 distinct restriction enzymes that have been routinely used for phytoplasma 16S rDNA RFLP analysis (Lee *et al*., 1998). After *in silico* restriction digestion, a virtual gel electrophoresis image was plotted and captured as a device-independent file in the PDF format. A Perl script was developed for pair-wise comparison of virtual RFLP patterns and for automated calculation of pattern similarity coefficient [$F = 2N_{xy}/(N_x+N_y)$].

Results and discussion

Cladistic and virtual RFLP analysis of over 900 available phytoplasma 16S rDNA sequences revealed that phytoplasma strains can be classified into 28 16Sr groups based on their cladistic positions and their distinctive RFLP pattern types (Wei *et al*., 2007). Of the more than 900 phytoplasma 16S rDNA entries, 27 be-

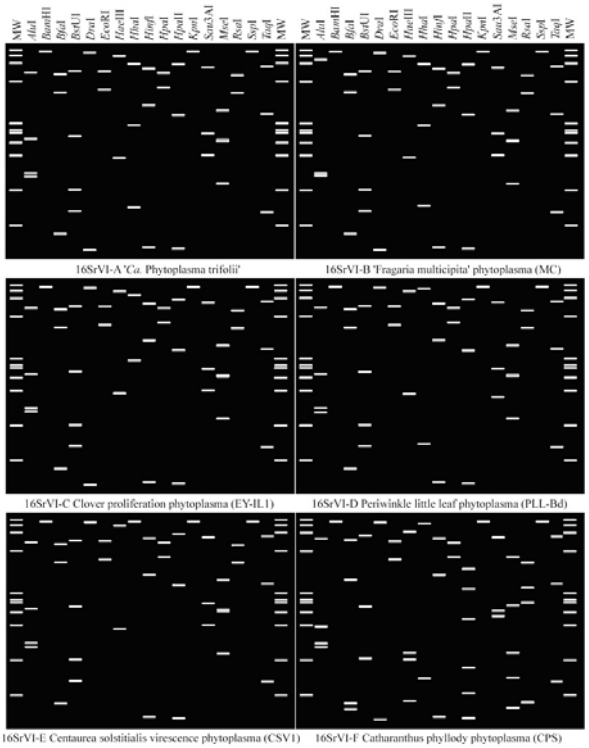

Figure 1. Virtual RFLP patterns from *in silico* digestions of 16S rDNA F2nR2 fragments. Patterns of six phytoplasma strains representing three previously and three newly delineated 16SrVI subgroups are shown. Recognition sites for the following 17 restriction enzymes were used in the simulated digestions: *Alu*I, *Bam*HI, *Bfa*I, *Bst*UI (*Tha*I), *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Sau*3AI (*Mbo*I), *Mse*I, *Rsa*I, *Ssp*I, and *Taq*I. MW: φX174 DNA-*Hae*III digestion.

long to the group 16SrVI, and they form a separate subclade in the phytoplasma phylogenetic tree (data not shown). *In silico* restriction digestion and gel plotting of the 27 16SrVI entries yielded 12 RFLP pattern types.

Of the 12 pattern types, three represent standard patterns of the three previously delineated 16SrVI subgroups. These include clover proliferation phytoplasma CP subgroup (16SrVI-A), '*Fragaria multicipita*' phytoplasma MC subgroup (16SrVI-B) and clover proliferation phytoplasma EY-IL1 subgroup (16SrVI-C) (figure 1). One or more new pattern type(s) identified in the current study may represent heterogeneous *rrn* operons in existing 16SrVI subgroups. However, the identification of a total of nine new pattern types clearly points to the presence of new subgroups in the 16SrVI phytoplasma group. Based on distinctive RFLP banding patterns and calculated pattern similarity coefficient, at least three new 16SrVI subgroups can be proposed, i.e. periwinkle little leaf phytoplasma PLL-Bd subgroup (16SrVI-D), *Centaurea solstitialis* virescence phytoplasma CSV1 subgroup (16SrVI-E), and catharanthus phyllody phytoplasma CPS subgroup (16SrVI-F) (figure 1). As shown in table 1, for each strain of a new subgroup, the similarity coefficient is less than 0.95 with strains in other subgroups.

Table 1. Similarity coefficients derived from 16S rDNA F2nR2 fragment virtual RFLP patterns.

Subgroup/ Strain	A	B	C	D	E	F
16SrVI-A (AY390261)	1.00					
16SrVI-B (AF190224)	0.97	1.00				
16SrVI-C (AF409070)	0.97	0.93	1.00			
16SrVI-D (AF228053)	0.92	0.88	0.90	1.00		
16SrVI-E (AY270156)	0.93	0.89	0.94	0.94	1.00	
16SrVI-F (EF186819)	0.90	0.87	0.87	0.82	0.83	1.00

Clover proliferation disease of alsike clover was first reported in the early 1960s as a yellows type virus disease. It was not until the mid-1970s that phytoplasma was discovered as the etiological agent of the disease (Hiruki and Wang, 2004). Since then, many emerging and previously unknown diseases have been found to be associated with infection by various phytoplasma strains of clover proliferation group. Existence of multiple 16S rDNA RFLP pattern types further demonstrates the genetic diversity and presumably biological complexity of the clover proliferation group phytoplasmas.

References

GUNDERSEN D. E., LEE I.-M., 1996.- Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs.- *Phytopathologia Mediterranea,* 35: 144-151.

HIRUKI C., WANG K. R., 2004.- Clover proliferation phytoplasma: '*Candidatus* Phytoplasma trifolii'.- *International Journal of Systematic and Evolutionary Microbiology,* 54: 1349-1353.

LEE I.-M., DAVIS R. E., GUNDERSEN-RINDAL D. E., 2000.- Phytoplasma: phytopathogenic mollicutes.- *Annual Review of Microbiology,* 54: 221-255.

LEE I.-M., HAMMOND R. W., DAVIS R. E., GUNDERSEN D. E., 1993.- Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasmalike organisms.- *Phytopathology,* 83: 834-842.

MCCOY R. E., CAUDWELL A., CHANG C. J., CHEN T. A., CHIYKOWSKYI L. N., COUSIN M.-T., DALE DE LEEUW G. T. N., GOLINO D. A., HACKETT K. J., KIRKPTRICK B. C., MARWITZ R., PETZOLD H., SHINA R. H., SUGIURA M., WHITCOMB R. F., YANG I. L., ZHU B. M., SEEMÜLLER E., 1989.- Plant diseases associated with mycoplasmalike organisms, pp. 545-560. In: *The Mycoplasmas*, *Vol 5* (WHITCOMB R. F., TULLY J. G., Ed.).- Academic Press, New York, USA.

TSAI J. H., 1979.- Vector transmission of mycoplasmal agents of plant diseases, pp. 265-307. In: *The Mycoplasmas III, Plant and Insect Mycoplasmas* (WHITCOMB R. F., TULLY J. G., Ed.).- Academic Press, New York, USA.

WEI W., DAVIS R. E., LEE I.-M., ZHAO Y., 2007.- Computer-simulated RFLP analysis of 16S rRNA genes: identification of ten new phytoplasma groups.- *International Journal of Systematic and Evolutionary Microbiology,* 57: 1855-1867.

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