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Differential amplification of sequence heterogeneous ribosomal RNA genes and classification of the ‘*Fragaria multicipita*’ phytoplasma

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Abstract

Ribosomal (r) RNA interoperon sequence heterogeneity in the ‘*Fragaria multicipita*’ phytoplasma, a member of group 16SrVI, was initially observed in RFLP patterns of rDNA amplified in the polymerase chain reaction (PCR), and was confirmed through sequence analysis of cloned rDNA. Sequences from operons *rrnA* and *rrnB* were amplified in PCR primed by primer pair P1/P7 but from only *rrnA* in PCR primed by primer pair R16mF2/R16mR1. Preferential amplification of DNA from operon *rrnA* was explained by base mismatches between the R16mF2/R16mR1 primers and primer annealing sites in *rrnB*. The results revealed potential for classification of a phytoplasma into two different subgroups within a 16S rRNA group, if the phytoplasma’s 16S rRNA gene sequences are independently characterized. It is suggested that the rRNA operon containing species-specific signature sequence(s) should be specified, and where possible sequences from both 16S rRNA genes should be included, in descriptions of new ‘*Candidatus* Phytoplasma species’.

Key words: ‘*Candidatus* Phytoplasma species’, phylogeny, operon, nested PCR

Abbreviations: MC, ‘*Fragaria multicipita*’ phytoplasma strain ‘multicipita’

Introduction

Phytoplasmas are minute, cell wall-less bacterial parasites and pathogens of plants (Lee *et al.* 2000). Analysis of conserved gene sequences, including 16S ribosomal RNA genes, has provided important insights into phytoplasma phylogeny and evolution from an *Acholeplasma*-like ancestor (Gundersen *et al.* 1994). Restriction fragment length polymorphism (RFLP) analyses of polymerase chain reaction (PCR)-amplified 16S rDNA have delineated at least 15 major groups and over 40 subgroups, each group representing at least one putative species (Gundersen *et al.* 1994). The identities of these groups and subgroups are in good agreement with groups and subclades defined on the basis of sequence analyses of 16S rRNA genes (Lee *et al.* 1998; Schneider *et al.* 1993). This progress is giving rise to an emerging phytoplasma taxonomy and to the recognition of ‘*Candidatus* Phytoplasma species’ (Davis *et al.* 1997; Griffiths *et al.* 1999; Montano *et al.* 2000, Sawayanagi *et al.* 1999; White *et al.* 1998; Zreik *et al.* 1995), a provisional taxonomic nomenclature that is being utilized because phytoplasmas cannot be isolated in culture and formal recognition of new species within the class Mollicutes requires descriptions of the organisms in pure culture.

The phytoplasma genome contains two rRNA operons which have appeared to be identical in a given phytoplasma (Schneider and Seemüller 1994; Smart *et al.* 1996). However, RFLP patterns (Davis *et al.* 1998b; Lee *et al.* 1998; Marcone *et al.* 2000) and nucleotide sequence data have indicated the presence of two diffe-

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The GenBank accession numbers for the phytoplasma strain ‘multicipita’ sequences determined in this study are AF190224 and AF190225.

rent 16S rRNA gene sequences in some phytoplasma strains (Jomantiene *et al.* 2002; Liefting *et al.* 1996). Although it is not known just how widespread rRNA interoperon sequence heterogeneity is among phytoplasmas, we hypothesize that it is common. Since phytoplasma subgroups (putative taxa) that are classified within major 16S rRNA RFLP groups are distinguished on the basis of differences in one or few restriction sites in 16S rDNA (Lee *et al.* 1998) and 'Candidatus Phytoplasma species' have been designated on the basis of 16S rRNA gene sequences, it is important to consider rRNA interoperon sequence heterogeneity when classifying newly discovered phytoplasmas and naming species.

In this communication we report the discovery and analysis of rRNA interoperon sequence heterogeneity in 'Fragaria multicipita' ('multicipita', MC) phytoplasma, the first example of interoperon heterogeneity in phylogenetic group 16SrVI (group VI, clover proliferation phytoplasma group). The 'multicipita' phytoplasma was previously found as the pathogen causing aberrant growth of *F. virginiana* in work that revealed the rare plant species '*F. multicipita*' as an invalid taxon (Jomantiene *et al.* 1998a). We also report the influence of PCR primer selection in preferential amplification of sequences from one of the two sequence heterogeneous rRNA operons in the phytoplasma genome. The findings in this study led us to propose that, where possible, sequences of both 16S rRNA genes be included in descriptions of 'Candidatus Phytoplasma species'. A preliminary synoptic report of these findings has been published (Davis *et al.*, 1998a).

Materials and methods

Phytoplasmas and plant samples. The 'Fragaria multicipita' phytoplasma ('multicipita' (MC) phytoplasma) was obtained from plants of *Fragaria chiloensis* that had been grafted with tissues from plants of 'Fragaria multicipita' (Fern.) kindly supplied by J. Postman (National Plant Germplasm Repository, Corvallis, OR). '*F. multicipita*' had been described as a rare species prior to work revealing that its unusual morphology was due to infection by a phytoplasma (Jomantiene *et al.* 1998a). Samples of symptomatic leaves were collected from infected plants and used in the extraction of DNA.

Polymerase chain reaction conditions and primers. Nucleic acid for use as template in the polymerase chain reaction (PCR) was extracted from fresh leaf tissue and purified as described (Jomantiene *et al.* 1998a).

Three pairs of oligonucleotides, phytoplasma universal primer pairs R16mF2/R16mR1 (mF2/R1) (Gundersen and Lee 1996), P1/P7 (Deng and Hiruki 1991; Smart *et al.* 1996), and R16F2n/R16R2 (F2n/R2)

(Gundersen and Lee 1996), were used to prime amplification of phytoplasma ribosomal RNA operon sequences in nested or direct PCRs. Two nested PCR protocols were used. In the first, DNA was initially amplified in PCR primed by mF2/R1 and was reamplified in PCR primed by F2n/R2, the latter which primes amplification of an approximately 1.2 kbp segment of the 16S rRNA gene. In the second, DNA was initially amplified in PCR primed by P1/P7 and was reamplified in PCR primed by F2n/R2. PCRs were carried out as previously described (Jomantiene *et al.* 1998b).

Restriction fragment length polymorphism (RFLP) analysis of amplified phytoplasma DNA. Products from PCR primed by F2n/R2 were analyzed by single enzyme digestion, according to endonuclease manufacturers' instructions, with *AluI*, *HhaI*, and *KpnI* (Invitrogen/Life Technologies, Carlsbad, CA), and *DdeI*, *DraI*, *HaeIII*, *HinfI*, *HpaI*, *HpaII*, *MseI*, *RsaI*, *Sau3AI*, and *TaqI*, (New England Biolabs, Beverly, MA). The RFLP profile of digested DNA was analyzed as previously (Jomantiene *et al.* 1998b). DNA fragment size standards used were PhiX174 RF *HaeIII* digest (Life Technologies) and 1.6 kb ladder (Fermentas AB, Vilnius, Lithuania). RFLP patterns were compared with those previously published (Davis *et al.*, 1997; Jomantiene *et al.* 1998a,b, 2002; Lee *et al.* 1998). Phytoplasma 16S rRNA group and subgroup designations are according to the classification scheme of Lee *et al.* (1998) and are thus based upon RFLP and putative restriction site patterns of a 1.2 kbp 16S rDNA sequence corresponding to that amplified in PCR primed by primer pair F2n/R2.

Cloning of PCR products and screening of cloned rRNA operon sequences. We cloned phytoplasmal 1.8 kbp rDNA products, that had been amplified in direct PCRs primed by P1/P7, using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Recombinant plasmids were screened for the presence of cloned rRNA operon fragments by amplification of DNA from recombinant plasmids in PCRs primed by F2n/R2 followed by RFLP analysis of the amplified DNA. The RFLP profiles were compared with those obtained from analysis of uncloned PCR products.

Nucleotide sequencing and putative restriction site analysis. Cloned DNA sequences (1.8 kbp) from the phytoplasmal rRNA operons were sequenced using automated DNA sequencing. The nucleotide sequences determined in this study were deposited in the GenBank data library. Putative restriction site maps were generated by using DNASTAR program MapDraw option (DNASTAR, Inc., Madison, Wisc). Alignments of sequence pairs were generated and sequence similarities evaluated by using DNASTAR program Align option.

Phylogenetic analysis. 16S rRNA gene sequences (1.2 kb in size, representing the sequence between annealing sites of primer pair F2n/R2) from ‘multicipita’ phytoplasma and 29 other phytoplasma strains representing fifteen 16S rRNA phytoplasma groups (*sensu* Lee *et al.*, 1998), and *Acholeplasma laidlawii* and *A. palmae* were aligned using Clustal X version 1.63b (Thompson *et al.*,

1997) and DNASTAR. Analyses were performed with the computer program Clustal X. A phylogenetic tree was constructed by the Neighbor-Joining method and the tree was viewed by using TreeViewPPC (Page, 1996). *A. laidlawii* was selected as the outgroup to root the tree. GenBank accession numbers of the phytoplasma and *Acholeplasma* nucleotide sequences are given in Table 1.

Table 1. GenBank accession numbers of phytoplasma and acholeplasma 16S rRNA gene sequences used in this study.

Phytoplasma	Group-subgroup ¹	Accession no.
Aster yellows (AY1)	I-B	AF322644
Maize bushy stunt (MBS)	I-B	AF487779
Alfalfa stunt (AlfS)	I-B	AF177384
Michigan aster yellows (MIAY)	I-B	M30970
Clover phyllody (CPh)	I-C	
	rrnA ²	AF222065
	rrnB	AF222066
‘ <i>Candidatus</i> Phytoplasma aurantifolia’	II-B	U15442
‘ <i>Ca. P. japonicum</i> ’	Unc. ³	AB010425
Canada X-disease (CX)	III-A	L33733
Clover yellow edge (CYE-C)	III-B	AF175304
Clover yellow edge (CYE-L)	III-B	AF173558
Gaillardia phyllody (GaiPh)	III-B	AY049029
Soybean veinal necrosis (SVN)	III-B	AF177383
Dandelion virescence (DanVir)	III-P	
	rrnA	AF370119
	rrnB	AF370120
Cirsium white leaf (CirWL)	III-R	
	rrnA	AF373105
	rrnB	AF373106
Palm lethal yellows (LY)	IV-A	U18747
Coconut lethal decline (LDT)	IV-C	X80177
Elm yellows (EY1)	V-A	AF189214
Clover proliferation (CP)	VI-A	L33761
‘multicipita’ (MC)	VI-B	
	rrnA	AF190224
	rrnB	AF190225
‘ <i>Ca. P. fraxini</i> ’	VII-A	AF189215
Loofah witches’ broom (LfWB)	VIII-A	AF248956
Pigeon pea WB (PPWB)	IX-A	AF248957
Apple proliferation (APU)	X-A	AF248958
Rice yellow dwarf (RYD)	XI-A	D12581
Leafhopper borne BVK	XI-C	X76429
Stolbur (STOL)	XII-A	AF248959
‘ <i>Ca. P. australiense</i> ’	XII-B	L76865
Mexican periwinkle virescence (MPV)	XIII-A	AF248960
Bermudagrass whiteleaf (BGWL)	XIV-A	AF248961
‘ <i>Ca. P. brasiliense</i> ’	XV-A	AF147708
<i>Acholeplasma palmae</i>	N.A. ⁴	L33734
<i>A. laidlawii</i>	N.A.	M23932

¹ 16S rRNA RFLP group and subgroup affiliation of indicated phytoplasma. Group and subgroup assignments are based on RFLP analysis of DNA sequences corresponding to fragments amplified in PCRs primed by oligonucleotide pair R16F2n/R16R2, following the classification system of Lee *et al.* (1998).

² The two sequence heterogeneous rRNA operons in a phytoplasma are indicated as rrnA and rrnB, respectively.

³ Unc, unclassified.

⁴ N.A., not applicable.

Results

Influence of primer selection on amplification of rRNA operon sequences

Evidence for the presence of sequence heterogeneous 16S rRNA genes in the genome of 'multicipita' phytoplasma was first observed following RFLP analysis of PCR-amplified DNA. Although the RFLP analysis was done on DNA reamplified in the nested PCR, which was primed by F2n/R2, recognition of the sequence heterogeneity depended upon primer sequences selected for initial DNA amplification in the nested PCR protocol.

In the first three experiments (experiments 1, 2, and 3), we used the nested PCR protocol in which initial DNA amplification was primed by primer pair mF2/R1 and DNA reamplification was primed by primer pair F2n/R2. Following RFLP analysis using thirteen restriction enzymes including *TaqI*, the total of DNA fragment sizes in each RFLP pattern agreed with the 1.2 kbp size expected for 16S rDNA amplified in PCR primed by F2n/R2 and were the same as noted previously (Jomantiene *et al.* 1998a) (Fig. 1, mF2/R1 lanes 1,2,3; and data not shown). In agreement with previous work using this nested PCR protocol (Jomantiene *et al.* 1998a), these results gave no evidence for the presence of sequence heterogeneous 16S rRNA genes in 'multicipita' phytoplasma.

In the next three experiments (experiments 4, 5, and 6), initial DNA amplification was primed by primer pair P1/P7 and DNA reamplification was primed by primer pair F2n/R2. In these experiments, thirteen restriction enzyme RFLP patterns of 16S rDNA were the same as those obtained when initial DNA amplification was primed by mF2/R1, with one exception. In each of the experiments, the total of DNA fragment sizes in the *TaqI* RFLP pattern exceeded the 1.2 kbp size expected for 16S rDNA amplified in PCR primed by F2n/R2 (see Fig. 1, lanes P1/P7 4, 5, and 6). It was evident that this *TaqI* RFLP pattern did not result from incomplete digestion of substrate DNA by the enzyme, since control DNAs were completely digested by the enzyme, and repeated *TaqI* RFLP analyses of the same test phytoplasma DNA sample, and of DNA samples from 'multicipita' phytoplasma infected plants from other locations, yielded the same *TaqI* RFLP pattern (data not shown). We interpreted this RFLP pattern to be evidence of 16S rRNA interoperon sequence heterogeneity in 'multicipita' phytoplasma.

We interpreted the results of these six experiments to indicate that DNA from only one, or predominantly from one, of the two rRNA operons in 'multicipita' phytoplasma had been amplified in nested PCRs in which the first amplification was primed by mF2/R1 (Fig. 1, mF2/R1 lanes 1, 2, and 3), while DNA from both rRNA

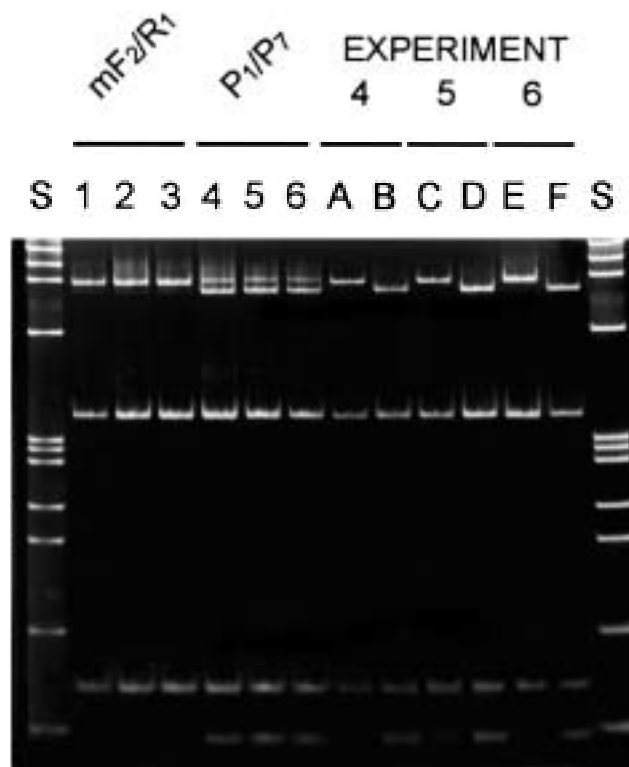


Fig. 1. RFLP analysis of cloned and uncloned rRNA operon sequences from 'multicipita' (MC) phytoplasma. DNA was amplified from the 'multicipita' phytoplasma in nested PCRs in six separate experiments. In experiments mF2/R1 1, 2, and 3, the first round of PCR was primed by R16mF2/R16mR1. In experiments P1/P7 4, 5, and 6, the first round of PCR was primed by P1/P7. In all 6 experiments, DNA was reamplified in a second, nested PCR primed by R16F2n/R16R2, and the products were digested with restriction endonuclease *TaqI* (lanes mF2/R1 1, 2, and 3; and lanes P1/P7 4, 5, and 6). Note that RFLP patterns in lanes P1/P7 4, 5, and 6 (but not RFLP patterns in lanes mF2/R1 1, 2, and 3) give evidence for the presence of two sequence heterogeneous DNAs. The DNA products of PCRs primed by P1/P7 in experiments P1/P7 4, 5, and 6 were cloned, and the cloned rDNAs were analyzed by *TaqI* digestion of 16S rDNA fragments that were amplified from six recombinant plasmids in PCR primed by F2n/R2 (lanes A-F). Two RFLP classes of cloned 16S rRNA gene sequences were observed. Lanes A-F, six cloned rDNAs, two from each of three cloning experiments; these lanes illustrate two RFLP classes that represent the class I RFLP pattern of operon *rrnA* (lanes A, C, and E) and the class II RFLP pattern of *rrnB* (lanes B, D, and F), respectively. S, Phi X174 *HaeIII* digest, size standard.

operons was amplified in PCRs primed by P1/P7 (Fig. 1, P1/P7 lanes 4, 5, and 6). Thus, the first evidence for 16S rDNA interoperon sequence heterogeneity depended upon whether the initial DNA amplification was primed by mF2/R1 or by P1/P7.

Separation and RFLP analysis of cloned rRNA operon sequences

The 1.8 kbp DNA products from the initial (P1/P7-primed) PCR amplifications of rDNA in experiments 4, 5, and 6 were cloned. The clones were analyzed by RFLP analysis of 1.2 kbp DNA fragments amplified from the cloned DNA inserts in PCRs primed by F2n/R2. Fig. 1 (lanes labeled A through F) shows results from *TaqI* RFLP analysis of the cloned rDNA inserts. The three separate experiments yielded the same results; two classes of *TaqI* RFLP patterns were observed. The class I pattern is seen in Fig. 1, lanes A, C and E, and the class II pattern is seen in Fig. 1, lanes B, D, and F. Visual superimposition of the two classes of RFLP pattern (for example, Fig. 1 lanes A and B) yielded a composite that was indistinguishable from the *TaqI* RFLP pattern obtained using uncloned PCR products (for example, Fig. 1 P1/P7 lane 4). These results indicated that the cloned 1.8 kbp sequences were derived from two sequence heterogeneous rRNA operons that had been amplified in PCRs primed by P1/P7. Operon rrnA is represented by the RFLP patterns in lanes 4A, 5C, and 6E in Fig. 1, and operon rrnB is represented by lanes 4B, 5D, and 6F, respectively.

Further comparisons of RFLP patterns of cloned and uncloned sequences revealed the identity of the sequence that had been amplified in PCRs primed by mF2/R1. The RFLP pattern of DNA amplified in the mF2/R1-F2n/R2 nested PCR protocol (Fig. 1, mF2/R1 lanes 1, 2, and 3) was indistinguishable from the class I RFLP pattern of cloned DNA shown in Fig. 1 (lanes 4A,

5C, and 6E). On the basis of these results, we concluded that the DNA sequence that had been amplified in PCR primed by mF2/R1 was derived from 'multicipita' phytoplasma operon rrnA.

Nucleotide sequence and putative restriction site analyses of cloned rRNA operon sequences

Nucleotide sequences were determined for rDNA clones represented by lanes 4A (clone MC9, operon rrnA) and 4B (clone MC10, operon rrnB), and the sequences have been deposited in the GenBank database. GenBank Accession numbers of the sequences are listed in Table 1. The nucleotide sequences were aligned to estimate the level of rRNA interoperon sequence similarity. The cloned 1.8 kbp sequences of rrnA and rrnB differed by five base substitutions located in the 16S rRNA gene. Results from analysis of putative restriction sites in the sequenced DNAs (Fig. 2) were in agreement with the fragment sizes obtained by enzymatic RFLP analysis of the amplified DNAs. Operon rrnA contains a *HpaII* site, not shared by rrnB, that is outside of the 1.2 kbp DNA amplified in PCR primed by F2n/R2 and was therefore not observed in the enzymatic RFLP analyses. Operon rrnB contains a *TaqI* site, within the 1.2 kbp DNA segment used for phytoplasma classification, that is absent in rrnA. Paired alignments with rrnA and rrnB revealed that a previously reported 'multicipita' phytoplasma 16S rDNA sequence (GenBank Acc. no. AF036354) (Jomantiene *et al.* 1998a) was derived from rrnA (data not shown).

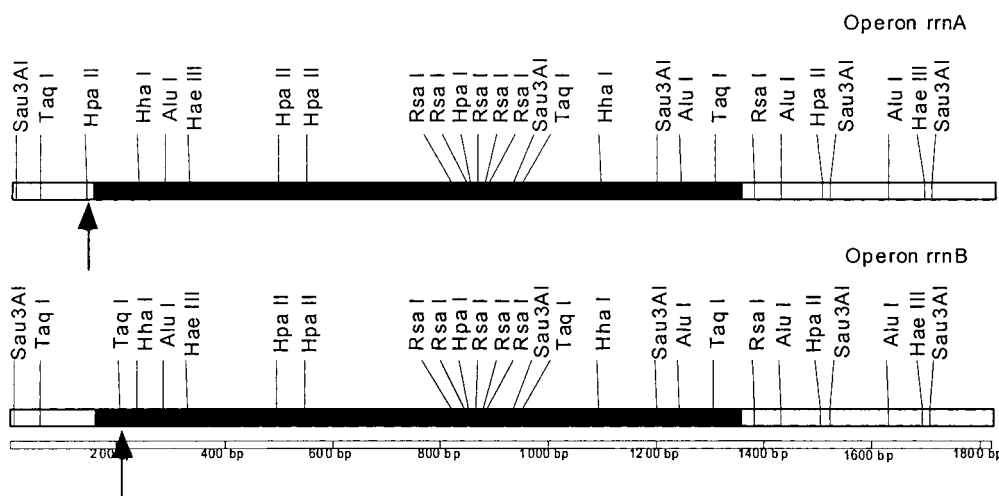


Fig. 2. Analysis of putative restriction sites in cloned DNA sequences from rRNA operons of the '*Fragaria multicipita*' ('multicipita', MC) phytoplasma. Operon rrnA, rRNA operon A. Operon rrnB, rRNA operon B. The solid bar represents the 1.2 kbp DNA sequence amplified in PCR primed by primer pair F2n/R2 (R16F2n/ R16R2). Arrows indicate sites that distinguish the sequences from one another.

Sequence heterogeneous rRNA operons in classification of 'multicipita' phytoplasma

To classsify the 'multicipita' phytoplasma in the grouping system of Lee *et al.* (1998), we compared the RFLP and putative restriction site patterns of 16S rDNA from operons *rrnA* and *rrnB*, respectively, with RFLP patterns published for previously classified phytoplasmas. The results indicated that the 'multicipita' phytoplasma should be classified in group 16SrVI (group VI, clover proliferation phytoplasma group), whether *rrnA* or *rrnB* is used as the basis for classification. However, it was evident that subgroup classification would differ depending upon which operon were used for classification. Using the 16S rDNA of *rrnA* for classification clearly placed the 'multicipita' phytoplasma in subgroup VI-B, in agreement with previous work (Jomantiene *et al.* 1998a). Using the 16S rDNA of *rrnB* would necessitate placement of the 'multicipita' phytoplasma in a distinct new subgroup (VI-C), due to the unique collective RFLP and putative restriction site patterns of its 16S rDNA. Thus, we base the classification on *rrnA*, retaining the 'multicipita' phytoplasma as the only known member of subgroup VI-B.

Phylogenetic analysis

On the basis of analysis of 16S rRNA gene sequences, we constructed a phylogenetic tree indicating relationships among previously known phytoplasmas and the 'multicipita' phytoplasma (Fig. 3). The branching order and branch lengths of the tree are consistent with the hypothesis that *rrnA* and *rrnB* in the 'multicipita' phytoplasma evolved from a common ancestral sequence. This observation is more evident in the rRNA operons of "multicipita" phytoplasma than in the sequence heterogeneous rRNA operon pairs of *cirsium* yellows and dandelion virescence phytoplasmas (Fig. 3). Inclusion of the two 16S rRNA genes from 'multicipita' phyto-

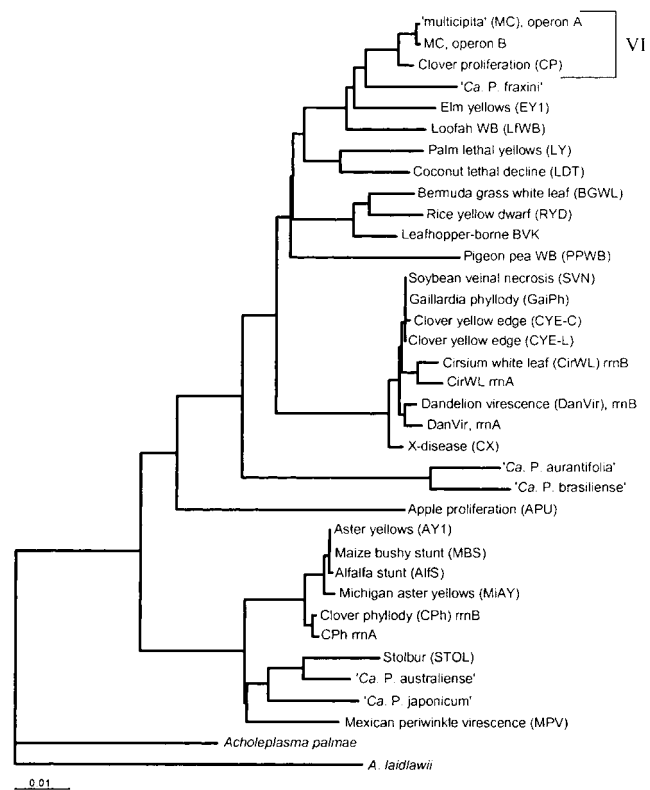


Fig. 3. Phylogenetic tree constructed by the Neighbor-Joining method of 16S rRNA gene sequences from 30 phytoplasmas and *Achleoplasma laidlawii* and *A. palmae*, employing *A. laidlawii* as the outgroup. Roman numeral VI indicates group VI (16SrVI, clover proliferation phytoplasma group).

plasma reinforces the clear distinction of group 16SrVI from all other phytoplasmas in the tree. The phylogenetic position of the 'multicipita' phytoplasma supports its recognition as representative of a distinct lineage within the group VI phytoplasma subclade.

Table 2. Matches and mismatches between PCR primers and primer annealing sites in the two rRNA operons of 'multicipita' phytoplasma

Primer	Nucleotide sequences of primer annealing sites in indicated operon*	
	<i>rrnA</i>	<i>rrnB</i>
R16mF2	5'-CATGCAAGTCGAACGGA-3'	5'-CATGCAAGTCGAACGaA-3'
R16mR1	5'-CTTAACCCCAATCATCaAC-3'	5'-CTTAACCCCAATCATCaAC-3'
R16F2n	5'-GAAACGgtTGCTAAGACTGG-3'	5'-GAAACGgtTGCTAAGACTGG-3'
R16R2	5'-TGACGGGCGGTGTGTACAAACCCCG-3'	5'-TGACGGGCGGTGTGTACAAACCCCG-3'

* Phytoplasma DNA bases matching the corresponding primer sequence are in capital letters; base mismatches are in lower case letters. Nucleotide sequences of the primers are as follows: R16mF2, 5'-CATGCAAGTCGAACGGA-3'; R16mR1, 5'-CTTAACCCCAATCATCGAC-3'; R16F2n, 5'-GAAACGACTGCTAAGACTGG-3'; R16R2, 5'-TGACGGGCGGTGTGTACAAACCCCG-3' (Gundersen & Lee, 1996).

Primer selection and primer annealing sites in phytoplasmal rRNA operons

To explain failure of the mF2/R1-F2n/R2 nested PCR protocol to yield DNA amplification from both rRNA operons, we hypothesized the existence of significant mismatch(es) between primer pair mF2/R1 and the rrnB sequence. Table 2 summarizes base matches and mismatches between PCR primers and annealing sites in the 'multicipita' phytoplasma rDNA. Whereas primer R1 (mR1, R16mR1) was mismatched in rrnA at a single base position near the primer's 3'-end, and primer mF2 (R16mF2) was perfectly matched in rrnA, both of these primers were mismatched with rrnB near their 3'-ends (Table 2), yielding initial DNA amplification only from rrnA. Nested primer R2 (R16R2) was perfectly matched with sequences in both operons, and nested primer F2n (R16F2n) was mismatched with both operons at the relatively distant 13th and 14th bases from the primer's 3'-end, permitting DNA reamplification from both operons when initial amplification was primed by P1/P7.

Discussion

In this study, phylogenetic analysis based on nucleotide sequences of both sequence heterogeneous 16S rRNA genes has indicated 'multicipita' phytoplasma to represent a distinct lineage whose ribosomal RNA operons rrnA and rrnB evolved from a common ancestral sequence. Previously, RFLP patterns have also been interpreted as evidence of rRNA interoperon heterogeneity in some group 16SrI phytoplasmas (Lee *et al.* 1998; Marcone *et al.* 2000). However, few previous studies have documented phytoplasmal 16S rRNA interoperon sequence heterogeneity on the basis of nucleotide sequence data (this paper; Jomantiene *et al.* 2002; Liefting *et al.* 1996). Current RFLP and sequence data indicate 16S rRNA interoperon sequence heterogeneity in phytoplasma groups 16SrVI, 16SrI, 16SrIII, and 16SrXII (this study; Lee *et al.* 1998; Jomantiene *et al.* 2002; Liefting *et al.* 1996). Occurrence of 16S rRNA interoperon heterogeneity in such divergent lineages leads us to hypothesize that it is a common phenomenon in the phytoplasma genome.

While a few rRNA interoperon base substitutions may not alter phylogenetic position (Liefting *et al.* 1996), we suggest that 16S rRNA interoperon sequence heterogeneity should be considered in the development of phytoplasma classification schemes and taxonomy. For example, RFLP analysis of a phytoplasma's sequence heterogeneous rRNA operons in mutual isolation could result in erroneous assignment of the same phytoplasma to two different 16S rRNA subgroups, or putative taxa, in current classification schemes that are based

on RFLP patterns. The present findings are the first to indicate the significance of primer selection in differential amplification of heterogeneous rRNA operon sequences in a phytoplasma genome. Since certain primer pairs prime the amplification of DNA from both sequence heterogeneous rRNA operons in a phytoplasma genome (this study), it also follows that nucleotide sequencing of the product from such a PCR would be carried out on a mixture of rDNA sequences. This raises the possibility that a unique 16S rDNA sequence that forms part of a phytoplasma taxonomic description could conceivably be a consensus that contains bases from both of two heterogeneous operons if the sequence were based on PCR-amplified, uncloned DNA. The emerging phytoplasma taxonomy potentially could be impacted further, if a unique nucleotide sequence that forms part of a '*Candidatus* Phytoplasma species' description is found in only one of its two rRNA operons. Characterization of cloned DNA from both rRNA operons of a phytoplasma would help to resolve such issues. Thus, it is suggested that interoperon heterogeneous 16S rDNAs be sequenced after separation by cloning and that "*Ca. P. species*" descriptions specify the operon containing the species-specific signature sequence(s).

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References

- Davis, R. E., Dally, E. L., Gundersen, D. E., Lee, I.-M., Habili, N. (1997): "*Candidatus* Phytoplasma australiense," a new phytoplasma taxon associated with Australian grapevine yellows. *Int. J. Syst. Bacteriol.* **47**, 262–269.
- Davis, R. E., Jomantiene, R., Dally, E. L. (1998a): Interoperon sequence heterogeneity and differential PCR-mediated amplification of sequences from the two rRNA operons in phytoplasma. In: Proceedings of the 12th International Conference, International Organization of Mycoplasmaology, Sydney, Australia, 176–177.
- Davis, R. E., Jomantiene, R., Dally, E. L., Wolf, T. K. (1998b): Phytoplasmas associated with grapevine yellows in Virginia belong to group 16SrI, subgroup A (tomato big bud phytoplasma subgroup), and group 16SrIII, new subgroup I. *Vitis* **37**, 131–137.
- Deng, S., Hiruki, C. (1991): Amplification of 16S rRNA genes from culturable and nonculturable mollicutes. *J. Microbiol. Meth.* **14**, 53–61.
- Griffiths, H. M., Sinclair, W. A., Smart, C. D., Davis, R. E. (1999): The phytoplasma associated with ash yellows and

- lilac witches'-broom: "*Candidatus* Phytoplasma fraxini". Int. J. Syst. Bacteriol. **49**, 1605–1614.
- Gundersen, D. E., Lee, I.-M. (1996): Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. Phytopath. Mediterr. **35**, 144–151.
- Gundersen, D. E., Lee, I.-M., Rehner, S. A., Davis, R. E., Kingsbury, D. T. (1994): Phylogeny of mycoplasma-like organisms (phytoplasmas): a basis for their classification. J. Bacteriol. **176**, 5244–5254.
- Jomantiene, R., Davis, R. E., Valiunas, D., Alminaitė, A., Stanilius, J. (2002): New group 16SrIII phytoplasma lineages in Lithuania exhibit rRNA interoperon sequence heterogeneity. Europ. J. Plant Pathol. **108**, 507–517.
- Jomantiene, R., Davis, R. E., Dally, E. L., Maas, J. L. (1998a): The distinctive morphology of "*Fragaria multicapita*" is due to phytoplasma. HortScience **33**, 1069–1072.
- Jomantiene, R., Davis, R. E., Maas, J., Dally, E. L. (1998b): Classification of new phytoplasmas associated with diseases of strawberry in Florida based on analysis of 16S rRNA and ribosomal protein gene operon sequences. Int. J. Syst. Bacteriol. **48**, 269–277.
- Lee, I.-M., Gundersen-Rindal, D. E., Davis, R. E., Bartoszyk, I. (1998): Revised classification scheme of phytoplasmas based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences. Int. J. Syst. Bacteriol. **48**, 1153–1169.
- Lee, I.-M., Davis, R. E., Gundersen-Rindal, D. E. (2000): Phytoplasma: Phytopathogenic Mollicutes. Annu. Rev. Microbiol. **54**, 221–255.
- Liefting, L. W., Andersen, M. T., Beever, R. E., Gardner, R. C., Forster, R. L. S. (1996): Sequence heterogeneity in the two 16S rRNA genes of *Phormium* yellow leaf phytoplasma. Appl. Environ. Microbiol. **62**, 3133–3139.
- Marcone, C., Lee, I.-M., Davis, R. E., Ragozzino, A., Seemüller, E. (2000): Classification of aster yellows-group phytoplasmas based on combined analysis of rRNA and *tuf* gene sequences. Int. J. Syst. Evol. Microbiol. **50**, 1703–1713.
- Montano, H. G., Davis, R. E., Dally, E. L., Hogenhout, S., Pimentel, J. P., Brioso, P. S. T. (2000): "*Candidatus* Phytoplasma brasiliense", a new phytoplasma taxon associated with hibiscus witches' broom disease. Int. J. Syst. Evol. Microbiol. **51**, 1109–1118.
- Page, R. D. (1996): TREEVIEW: an application to display phylogenetic trees on personal computers. CABIOS **12**, 357–358.
- Sawayanagi, T., Horikoshi, N., Kanehira, T., Shinohara, M., Bertaccini, A., Cousin, M.-T., Hiruki, C., Namba, S. (1999): '*Candidatus* Phytoplasma japonicum', a new phytoplasma taxon associated with Japanese *Hydrangea* phyllody. Int. J. Syst. Bacteriol. **49**, 1275–1285.
- Schneider, B., Ahrens, U., Kirkpatrick, B. C., Seemüller, E. (1993): Classification of plant-pathogenic mycoplasma-like organisms using restriction-site analysis of PCR-amplified 16S rDNA. J. Gen. Microbiol. **139**, 519–527.
- Schneider, B., Seemüller, E. (1994): Presence of two sets of ribosomal genes in phytopathogenic mollicutes. Appl. Environ. Microbiol. **141**, 173–185.
- Smart, C. D., Schneider, B., Blomquist, C. L., Guerra, L. J., Harrison, N. A., Ahrens, U., Lorenz, K.-H., Seemüller, E., Kirkpatrick, B. C. (1996): Phytoplasma-specific PCR primers based on sequences of the 16S–23S rRNA spacer region. Appl. Environ. Microbiol. **62**, 2988–2993.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., Higgins, D. G. (1997): The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. **25**, 4876–4882.
- White, D. T., Blackwall, L. L., Scott, P. T., Walsh, K. B. (1998): Phylogenetic positions of phytoplasmas associated with dieback, yellow crinkle, and mosaic diseases of papaya, and their proposed inclusion in "*Candidatus* Phytoplasma australiense" and a new taxon, "*Candidatus* Phytoplasma australasia". Int. J. Syst. Bacteriol. **48**, 941–951.
- Zreik, L., Carle, P., Bové, J. M., Garnier, M. (1995): Characterization of the mycoplasma-like organism associated with witches'-broom disease of lime and proposition of a *Candidatus* taxon for the organism, "*Candidatus* Phytoplasma aurantifolia". Int. J. Syst. Bacteriol. **45**, 449–453.