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l, G. H. (1991). Two genomic clusters among
ppl. Microbiol. **14,** 393–396.
L. N., Fletcher, J., Hiruki, C., and Schaff,
ification and classification of mycoplasmalike
ain cluster. *Phytopathology* **82,** 977–986.
dersen, D. E. (1993). Universal amplification
fication of mycoplasmalike organisms. *Phy-*

mb, R. F., and Razin, S. (1986). DNA probes
like organisms in plant and insects. *FEMS*

y. *In* "Mycoplasmas: Molecular Biology and
. R. Finch, and J. B. Basman, eds.), pp. 3–

Glaser, G. (1984). Dctection of mycoplasmas
Vitro **20,** 404–408.
. "Molecular Cloning, a Laboratory Manual,"
Cold Spring Harbor, NY.
lar typing methods. *In* "Diagnostic Molecular
. Tenover, and T. J. White, eds.), pp. 26–50.

onucleic acid sequences in *Mycoplasma geni-*
Int. J. Syst. Bacteriol. **36,** 426–430.
. (1988a). Distinction of species and strains of
gerprints with an rRNA gene probe. *J. Clin.*

D., and Razin, S. (1988b). Ribosomal RNA
n *Mycoplasma gallisepticum* and *M. synoviae.*

1988c). Elongation factor (Ef-Tu) gene probe
EMS Microbiol. Lett. **50,** 145–149.
netic and antigenic relatedness between *My-*
ae. Vet. Microbiol. **19,** 75–84.

E6

PHYLOGENETIC CLASSIFICATION OF PLANT PATHOGENIC MYCOPLASMA-LIKE ORGANISMS OR PHYTOPLASMAS

Bernd Schneider, Erich Seemueller, Christine D. Smart, and Bruce C. Kirkpatrick

General Introduction

Sequence analyses of evolutionarily conserved genes such as the 16S ribosomal RNA (rRNA) have provided a detailed picture of the diversity and phylogenetic relationships of prokaryotes (Olsen *et al.*, 1994). Phylogenetic analyses have provided a coherent framework for the classification of diverse taxa, including the Mollicutes (Weisberg *et al.*, 1989). Another very attractive aspect of this type of analysis is the ability to analyze these phylogenetic markers from nonculturable prokaryotes such as plant pathogenic mycoplasma-like organisms (MLOs). Detailed analyses of several MLO 16S rRNA genes (Gunderson *et al.*, 1994; Kuske and Kirkpatrick, 1992; Lim and Sears, 1989; Namba *et al.*, 1993; Seemuller *et al.*, 1994) led to the conclusion that MLOs were a unique, monophyletic group of mollicutes that deserved a more informative and appropriate taxonomic classification. Therefore, the term "phytoplasma" was proposed and it is now being used by many researchers, including the authors of this chapter.

The process of characterizing these phylogenetically conserved genes has been greatly facilitated by the use of polymerase chain reaction (PCR) technologies to amplify and sequence these molecules directly. This chapter presents a phytoplasma taxonomic scheme that is based on the analysis of two evolutionary markers: the 16S rRNA gene and the spacer region that separates the 16S from

Molecular and Diagnostic Procedures in Mycoplasmology, Vol. I

the 23S rRNA genes. Phytoplasma phylogenetic relationships established by full-length 16S rRNA sequences agree with phytoplasma groups established by spacer region analyses (Kirkpatrick *et al.*, 1994). The information presented in this chapter provides an outline of the procedures that are available for phylogenetically classifying an unknown phytoplasma strain. Many of the specific procedures, such as running and reading a sequencing gel, will not be discussed here and the reader is referred to any of the numerous molecular biology manuals for information concerning such procedures. The procedures presented here will allow those familiar with such technologies to apply them to the classification of an unknown phytoplasma.

Two approaches have been historically used to characterize phytoplasma 16S rRNA genes: restriction fragment length polymorphisms (RFLP) of PCR-amplified 16S rRNA genes and sequence analysis of full-length 16S rRNA genes. Information presented in this chapter also includes phytoplasma phylogenetic relationships based on RFLP analysis and sequence analysis of the 16/23S spacer regions. The procedures used for obtaining and analyzing this type of data are outlined next.

RFLP Analysis of PCR-Amplified Ribosomal DNA

Introduction

The first phylogenetically based classification of the phytoplasmas was based on RFLP analysis of 16S rDNA (Lee *et al.*, 1993; Schneider *et al.*, 1993). This rapid method can provide valuable information on the phylogenetic and taxonomic classification of an unknown phytoplasma. The procedure is based on PCR amplification of phytoplasma rDNA using primers that recognize all phytoplasma strains but which do not amplify products from healthy plants. The primers listed in Table I can be used to amplify most or all of the 16S rRNA gene and the entire 16/23S spacer region. RFLP analysis of the larger PCR fragments is more desirable because they contain more restriction sites, which in turn provides more information than the shorter PCR fragments. The amplification products are digested with frequently cutting restriction endonucleases such as *Alu*I or *Rsa*I. In some cases, further differentiation can be obtained by digesting with additional enzymes such as *Mse*I or *Hha*I (Lee *et al.*, 1993).

Materials

Template DNA isolated from plants using the "phytoplasma enrichment" method (see Chapter B2, this volume)
Thermocycler, such as the Perkin–Elmer (Norwalk, CT) Model 480
A 50-μl PCR mixture containing the following materials:

SEQUENCE OF OL

Primer name	
Primer 1	5′ AAGA
Primer 3	5′ GGAT
Primer 4	5′ GAAG
Primer 5	5′ CGGC
Primer 7	5′ CGTC

[a] These can be used to ar
gene and 16S/23S spacer reg

A mixture of all four dNTPs, fi
PCR primers for amplifying phyt
tion of each primer is 0.5 μ*M*
1× *Taq* DNA polymerase buffer
enzyme manufacturer)
50 ng of phytoplasma DNA (see
1 unit of *Taq* DNA polymerase
1 drop of mineral oil to cover re

Horizontal gel electrophoresis unit
Molecular biology grade electrophore
Electrophoresis buffer, such as Tris/b
Molecular size standards such as the
MD)
Ethidium bromide solution (1 μg/ml)
UV transilluminator
Restriction endonucleases such as *Alu*
Restriction enzyme buffer (supplied b
DNA samples from representative stra
clades
Reagents and equipment for polyacryla
this volume)

Procedure

PCR AMPLIFICATION OF PHYTOPLAS
SPACER REGIONS

Primers 1 and 7 are used to amplify
rRNA gene plus the 16/23S spacer re
toplasma DNA in the template is low,

TABLE I

SEQUENCE OF OLIGONUCLEOTIDE PRIMERS[a]

Primer name	Sequence
Primer 1	5′ AAGAGTTTGATCCTGGCTCAGGATT 3′
Primer 3	5′ GGATGGATCACCTCCTT 3′
Primer 4	5′ GAAGTCTGCAACTCGACTTC 3′
Primer 5	5′ CGGCAATGGAGGAAACT 3′
Primer 7	5′ CGTCCTTCATCGGCTCTT 3′

[a] These can be used to amplify and sequence the 16S rRNA gene and 16S/23S spacer region.

A mixture of all four dNTPs, final concentration is 150 μM
PCR primers for amplifying phytoplasma 16S rRNA genes, final concentration of each primer is 0.5 μM
1× *Taq* DNA polymerase buffer containing 1.5 mM $MgCl_2$ (supplied by the enzyme manufacturer)
50 ng of phytoplasma DNA (see above)
1 unit of *Taq* DNA polymerase
1 drop of mineral oil to cover reaction mixture

Horizontal gel electrophoresis unit
Molecular biology grade electrophoresis agarose
Electrophoresis buffer, such as Tris/borate/EDTA (TBE)
Molecular size standards such as the 1-kb ladder (GIBCO-BRL, Gaithersburg, MD)
Ethidium bromide solution (1 μg/ml)
UV transilluminator
Restriction endonucleases such as *Alu*I and *Rsa*I
Restriction enzyme buffer (supplied by the enzyme manufacturer)
DNA samples from representative strains in the major phylogenetic phytoplasma clades
Reagents and equipment for polyacrylamide gel electrophoresis (see Chapter E4, this volume)

Procedure

PCR AMPLIFICATION OF PHYTOPLASMA 16S rRNA AND 16/23S SPACER REGIONS

Primers 1 and 7 are used to amplify a PCR product that contains the entire 16S rRNA gene plus the 16/23S spacer region. However, when the amount of phytoplasma DNA in the template is low, as it often is in woody plant preparations,

better amplification may be achieved using primers 5 and 7. The sequences of all the phytoplasma-specific primers are listed in Table I and their relative positions are shown in Fig. 1.

Phytoplasma 16S rRNA genes and/or SRs are amplified from the plant DNA preparation using the following thermocycler conditions: initial strand denaturation for 5 minutes at 95°C, subsequent denaturing steps for 1 minute at 95°C, annealing for 1 minute at 53°C, and extension for 1.5 to 2 minutes (primers 1 or 5 and 7) at 72°C. An adequate amount of PCR product is usually obtained after 30 cycles; however, 35 cycles may be necessary in some very low-titer woody plants.

The quality and the quantity of the PCR product are assessed by electrophoresing approximately 5 μl of the reaction mixture on a horizonal agarose gel. The product is visualized under UV light after staining with the ethidium bromide solution. The amplified phytoplasma product obtained using primer 1 and 7 is approximately 1800 bp, whereas the product from 5 and 7 is approximately 1450 bp.

An appropriate amount (approximately 100 to 200 ng) of the PCR amplification product is digested with the previously described restriction enzymes at 37°C for 4 hours or overnight. The reaction is stopped by adding $\frac{1}{10}$ volume of 10× gel-loading buffer. Approximately one-third of the digest is electrophoresed on a 5 or 8% polyacrylamide gel. Alternatively, the digestion products may be electrophoresed in size-selection gels such as FMC Metaphor or NuSieve. The restriction fragments are visualized under UV light after staining with ethidium bromide, and the restriction profiles of the unknown samples are compared to reference strains. *Alu*I and *Rsa*I profiles from seven major RFLP groups and two subgroups (Schneider *et al.*, 1993) are shown in Fig. 2.

Discussion

RFLP profiles of PCR-amplified, 16S rDNA have been determined for a large number of phytoplasma strains (Lee *et al.*, 1993; Schneider *et al.*, 1993). Based on the similarities and differences of these profiles, several phytoplasma RFLP groups and subgroups were established. Comparisons showed that most of the RFLP groups correlated well with the major groups established by full-length 16S rDNA sequence analysis (Gunderson *et al.*, 1994; Seemuller *et al.*, 1994).

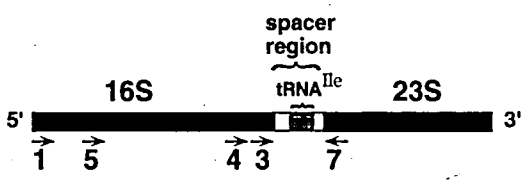


Fig. 1. Map of the 16S/23S rRNA operon found in phytoplasmas. The positions of oligonucleotide primers used in PCR analysis are represented as arrows.

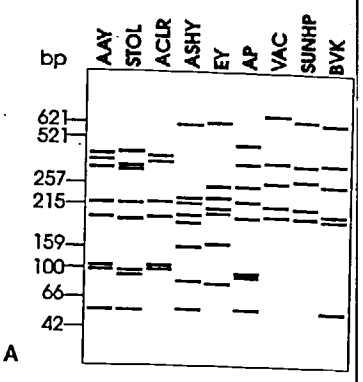


Fig. 2. *Alu*I (A) and *Rsa*I (B) restriction fragme toplasma rDNA amplified with primers 1 and groups and two subgroups established by Schne the strains are shown in Fig. 3. AAY, Americar stolbur—Serbia (subgroup of RFLP group I); A group II); ASHY, ash yellows—New York (RFL (RFLP group IV); AP, apple proliferation—Gerr broom—Germany (RFLP group VI); SUNHP, su RFLP group VI); BVK, leafhopper-borne phytop

However, there are a few cases where cl accurate as classification by sequence ACLR and AAY clearly differ in their r rDNA sequences are 98.7% homologou where two phytoplasma RFLP profiles those of strains ACLR and AAY, but seq be more distantly related. Despite these cessfully applied provided that the patt taxonomic status has been determined b RFLP pattern of an unknown phytoplasm can be properly classified. If the patter sequence analysis will be necessary to d tion of the unknown strain.

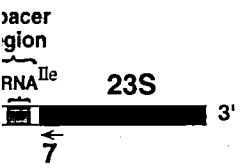
Phylogenetic Classification of P rRNA or 16/23S Spacer Region

Sequence analysis of the complete phy most accurate picture of phylogenetic rel

g primers 5 and 7. The sequences of all
d in Table I and their relative positions

SRs are amplified from the plant DNA
cler conditions: initial strand denatura-
lenaturing steps for 1 minute at 95°C,
sion for 1.5 to 2 minutes (primers 1 or 5
CR product is usually obtained after 30
ıry in some very low-titer woody plants.
product are assessed by electrophores-
ixture on a horizonal agarose gel. The
staining with the ethidium bromide solu-
obtained using primer 1 and 7 is approxi-
om 5 and 7 is approximately 1450 bp.
100 to 200 ng) of the PCR amplifica-
y described restriction enzymes at 37°C
stopped by adding $\frac{1}{10}$ volume of 10×
ird of the digest is electrophoresed on a
y, the digestion products may be elec-
s FMC Metaphor or NuSieve. The re-
UV light after staining with ethidium
he unknown samples are compared to
rom seven major RFLP groups and two
hown in Fig. 2.

DNA have been determined for a large
., 1993; Schneider *et al.*, 1993). Based
se profiles, several phytoplasma RFLP
Comparisons showed that most of the
ıajor groups established by full-length
ı *et al.*, 1994; Seemuller *et al.*, 1994).



nd in phytoplasmas. The positions of oligo-
resented as arrows.

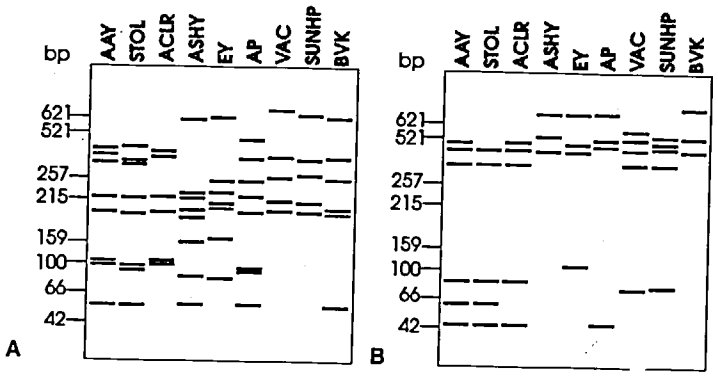


Fig. 2. *Alu*I (A) and *Rsa*I (B) restriction fragment length polymorphism (RFLP) profiles of phytoplasma rDNA amplified with primers 1 and 7. The strains represent the seven major RFLP groups and two subgroups established by Schneider *et al.* (1993). The phylogenetic positions of the strains are shown in Fig. 3. AAY, American aster yellows—Florida (RFLP group I); STOL, stolbur—Serbia (subgroup of RFLP group I); ACLR, apricot chlorotic leaf roll—Spain (RFLP group II); ASHY, ash yellows—New York (RFLP group III); EY, elm witches' broom—France (RFLP group IV); AP, apple proliferation—Germany (RFLP group V); VAC, vaccinium witches' broom—Germany (RFLP group VI); SUNHP, sunhemp witches' broom—Thailand (subgroup of RFLP group VI); BVK, leafhopper-borne phytoplasma—Germany (RFLP group VII).

However, there are a few cases where classification by RFLP analysis was not as accurate as classification by sequence analysis. For example, strains such as ACLR and AAY clearly differ in their restriction patterns (Fig. 2) but their 16S rDNA sequences are 98.7% homologous. In contrast, there are other instances where two phytoplasma RFLP profiles are less different from each other than those of strains ACLR and AAY, but sequence analysis showed the organisms to be more distantly related. Despite these problems, RFLP analysis can be successfully applied provided that the patterns are compared with strains whose taxonomic status has been determined by 16S rDNA sequence analysis. If an RFLP pattern of an unknown phytoplasma is identical to a standard strain then it can be properly classified. If the pattern differs from the standard stain then sequence analysis will be necessary to determine correctly the taxonomic position of the unknown strain.

Phylogenetic Classification of Phytoplasmas Based on 16S rRNA or 16/23S Spacer Region Sequence Analysis

Sequence analysis of the complete phytoplasma 16S rRNA gene provides the most accurate picture of phylogenetic relationships among phytoplasmas. How-

ever, it takes a considerable amount of time and effort to sequence both strands of the full-length 16S rRNA molecule (approximately 1540 nucleotides long). The spacer region (SR) that separates the 16S from the 23S rRNA also contains phylogenetically useful information and, since this region is approximately 280 nucleotide long, it is much easier to sequence. In addition, at the time this chapter was written, full-length 16S rRNA had been determined for approximately 32 phytoplasmas, whereas SR sequences had been determined for more than 60 phytoplasma strains. A manuscript describing the use of SR sequences for classifying phytoplasmas is now being prepared and the SR sequences will soon be available in GenBank.

Once the 16S rRNA or SR sequence is obtained, it is necessary to analyze it using one of the available phylogenetic programs on an appropriate computer. The sequence of the unknown phytoplasma is then compared with the sequences of known phytoplasmas that are available in databases such as GenBank.

Table I presents the sequences of several oligonucleotide primers that can be used to PCR amplify the 16S rRNA gene and/or the 16/23S spacer region. The locations of the primers listed in Table I are shown in Fig. 1.

Materials

See the previous section for materials and equipment required for PCR amplification of phytoplasma rDNA. In addition, the following materials are needed for DNA sequencing operations.

PCR product purification system, such as Wizard Clean Up Columns (Promega, Madison, WI)
Sequencing apparatus and power supply
Cycle sequencing kit, such as dsDNA cycle sequencing kit (GIBCO, BRL, Gaithersburg, MD)

Procedure

PCR AMPLIFICATION OF PHYTOPLASMA 16S rRNA AND 16/23S SPACER REGIONS

The PCR amplification of phytoplasma rDNA has been described earlier. In general, it is most desirable to use primers 1 and 7 to amplify a PCR product that contains the entire 16S rRNA gene plus the 16/23S spacer region, especially if one desires to sequence the 16S rRNA gene. However, if only the phytoplasma SR is to be sequenced, in some instances better amplification is obtained from low-titer hosts using primers 5 and 7 or primers 4 and 7 (Table I, Fig. 1). Phytoplasma 16S rRNA genes and/or SRs are amplified from the plant DNA

E6 Phylogenetic

T

GenBank Accession Numbe
from MLOs and C

Strain
Acholeplasma laidlawii
Acholeplasma modicum
Acholeplasma sp. J233
American aster yellows—Florida
Apricot chlorotic leaf roll—Spain
Apple proliferation—Germany
Ash yellows—New York
Aster yellows—California
Aster yellows—Japan
Aster yellows—Maryland
Aster yellows—Michigan
Black alder witches' broom—Germany
BVK—Germany
Canadian peach X-disease—Canada
Clover phyllody—Canada
Clover proliferation—Canada
Clover yellow edge—Canada
Elm witches' broom—France
European stone fruit yellows—Germany
Flavesence Doree—France
Loofah witches' broom—Taiwan
Palm lethal yellows—Florida
Peanut witches' broom—Taiwan
Pear decline—Germany
Pigeon pea witches' broom—Florida
Rice yellow dwarf—Japan
Stolbur—Serbia
Sugar cane white leaf—Thailand
Sunhemp witches' broom—Thailand
Sweet potato witches' broom—Taiwan
Tomato big bud—Arkansas
Tsuwabuki witches' broom—Japan
Vaccinium witches' broom—Germany
Vk grapevine yellows—Germany
Western X-disease—California

preparation using the following thermocy
tion for 5 minutes at 95°C, subsequent
annealing for 1 minute at 53°C, and exten
minutes (primers 1 and 7 or primer 5 a

TABLE II

GenBank Accession Numbers of 16S rRNA Gene Sequences from MLOs and Culturable Mollicutes

Strain	Accession No.	Reference
Acholeplasma laidlawii	M23933	Weisburg *et al.* (1989)
Acholeplasma modicum	M23933	Weisburg *et al.* (1989)
Acholeplasma sp. J233	L33734	Gunderson *et al.* (1994)
American aster yellows—Florida	X68373	Seemuller *et al.* (1994)
Apricot chlorotic leaf roll—Spain	X68383	Seemuller *et al.* (1994)
Apple proliferation—Germany	X68375	Seemuller *et al.* (1994)
Ash yellows—New York	X68339	Seemuller *et al.* (1994)
Aster yellows—California	M86340	Kuske and Kirkpatrick (1992)
Aster yellows—Japan	D12569	Namba *et al.* (1993)
Aster yellows—Maryland	L33767	Gunderson *et al.* (1994)
Aster yellows—Michigan	M30970	Lim and Sears (1989)
Black alder witches' broom—Germany	X76431	Seemuller *et al.* (1994)
BVK—Germany	X76429	Seemuller *et al.* (1994)
Canadian peach X-disease—Canada	L33733	Gunderson *et al.* (1994)
Clover phyllody—Canada	L33762	Gunderson *et al.* (1994)
Clover proliferation—Canada	L33761	Gunderson *et al.* (1994)
Clover yellow edge—Canada	L33766	Gunderson *et al.* (1994)
Elm witches' broom—France	X68376	Seemuller *et al.* (1994)
European stone fruit yellows—Germany	X68374	Seemuller *et al.* (1994)
Flavesence Doree—France	X76560	Seemuller *et al.* (1994)
Loofah witches' broom—Taiwan	L33764	Gunderson *et al.* (1994)
Palm lethal yellows—Florida		Unpublished
Peanut witches' broom—Taiwan	L33765	Gunderson *et al.* (1994)
Pear decline—Germany	X76425	Seemuller *et al.* (1994)
Pigeon pea witches' broom—Florida		Unpublished
Rice yellow dwarf—Japan	D12581	Namba *et al.* (1993)
Stolbur—Serbia	X76427	Seemuller *et al.* (1994)
Sugar cane white leaf—Thailand	X76432	Seemuller *et al.* (1994)
Sunhemp witches' broom—Thailand	X76433	Seemuller *et al.* (1994)
Sweet potato witches' broom—Taiwan	L33770	Gunderson *et al.* (1994)
Tomato big bud—Arkansas	L33733	Gunderson *et al.* (1994)
Tsuwabuki witches' broom—Japan	D12580	Namba *et al.* (1993)
Vaccinium witches' broom—Germany	X76430	Seemuller *et al.* (1994)
Vk grapevine yellows—Germany	X76428	Seemuller *et al.* (1994)
Western X-disease—California	L04682	Unpublished

preparation using the following thermocycler conditions: initial strand denaturation for 5 minutes at 95°C, subsequent denaturing steps for 1 minute at 95°C, annealing for 1 minute at 53°C, and extension for 1 minute (primers 4 and 7) or 2 minutes (primers 1 and 7 or primer 5 and 7) at 72°C. An adequate amount of

PCR product is almost always obtained after 30 cycles; however, 35 cycles may be necessary in some very low-titer woody plants.

The quality and the quantity of the PCR product are assessed by electrophoresing approximately 5 μl of the reaction mixture on a horizonal agarose gel as previously described. In most cases, there is usually a single band which corresponds to the phytoplasma product. If this is the case then the rest of the PCR reaction mixture is put through the PCR cleanup column to remove the unused primers and buffer. If multiple bands are observed then the reaction mixture will need to be electrophoresed in an agarose gel and the phytoplasma DNA band excised from the gel. The DNA is then removed from the gel matrix using standard procedures such as the GeneClean System (Bio101, LaJolla, CA).

SEQUENCING PCR-AMPLIFIED PHYTOPLASMA 16S rRNA OR 16/23S SPACER REGION

The purified, PCR-amplified phytoplasma 16S rRNA and/or spacer region can either be sequenced directly using a PCR cycle sequencing kit, such as dsDNA cycle sequencing kit (Gibco BRL) or be cloned into a PCR cloning vector, such as the TA cloning kit (Invitrogen, San Diego, CA), and sequenced by standard dideoxy chain-termination reactions. If the full-length 16S rRNA molecule is to be sequenced, then internal sequencing primers will need to be used. Several internal 16S rRNA primers, their sequences, and their locations in the 16S rRNA molecule are presented by Gunderson *et al.* (1994), Namba *et al.* (1993), and Seemuller *et al.* (1994). If the spacer region is to be sequenced then the PCR product can be readily sequenced in both directions using primers 7 and 3 (Table I).

SEQUENCE ANALYSIS OF PHYTOPLASMA 16S rRNA OR 16/23S SR

Spacer region or 16S rRNA sequences are compared and aligned with published sequences using a compilation program such as the Pileup program of the UW-GCG (University of Wisconsin). If the GCG program is used then gap weight should be set to 2.0 and the gap length set to 0.2. The aligned sequences of the known and unknown phytoplasmas can be simply compared and the percentage homology determined or, more informatively, the sequence can be analyzed using a phylogenetic analysis program such as Swofford's Phylogenetic Analysis Using Parsimony (PAUP) version 3.1.1 (1993). This program also contains "bootstrap" analysis which gives confidence values for the branches within the phylogenetic tree. If PAUP is used, bootstrap analysis can be performed using 100 replicates with 10 random additions of taxa per replicate, implementing the tree bisection and reconnection branch-swapping algorithm. Uninformative characters are ignored in this type of analysis.

Table II lists the GenBank accession numbers of the full-length phytoplasma

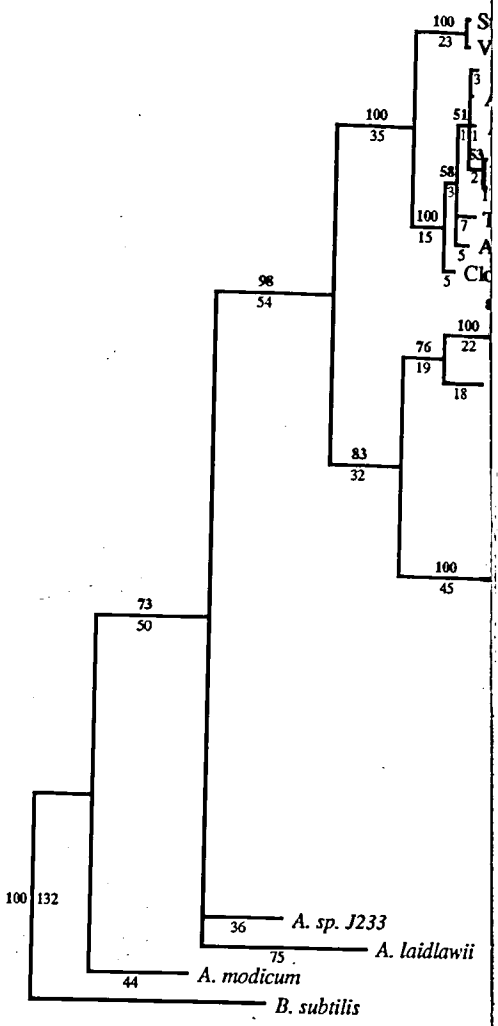


Fig. 3. Phylogram of 16S rRNA sequences g
(Swofford, 1993). Numbers in bold above the bra
numbers below the branches indicate branch len

16S rRNA sequences that were available a
representative phylogenetic tree based on t
sequences that were available at the time t
Fig. 3, whereas Fig. 4 shows the relatio
sequences.

after 30 cycles; however, 35 cycles may
ody plants.
R product are assessed by electrophores-
mixture on a horizonal agarose gel as
re is usually a single band which corre-
his is the case then the rest of the PCR
cleanup column to remove the unused
observed then the reaction mixture will
se gel and the phytoplasma DNA band
en removed from the gel matrix using
lean System (Bio101, LaJolla, CA).

LASMA 16S rRNA OR 16/23S

asma 16S rRNA and/or spacer region can
R cycle sequencing kit, such as dsDNA
cloned into a PCR cloning vector, such
Diego, CA), and sequenced by standard
the full-length 16S rRNA molecule is to
g primers will need to be used. Several
nces, and their locations in the 16S rRNA
et al. (1994), Namba *et al.* (1993), and
region is to be sequenced then the PCR
th directions using primers 7 and 3 (Ta-

MA 16S rRNA OR 16/23S SR

es are compared and aligned with pub-
ogram such as the Pileup program of the
If the GCG program is used then gap
length set to 0.2. The aligned sequences
smas can be simply compared and the
nore informatively, the sequence can be
program such as Swofford's Phylogenetic
ersion 3.1.1 (1993). This program also
ives confidence values for the branches
is used, bootstrap analysis can be per-
random additions of taxa per replicate,
econnection branch-swapping algorithm.
in this type of analysis.
numbers of the full-length phytoplasma

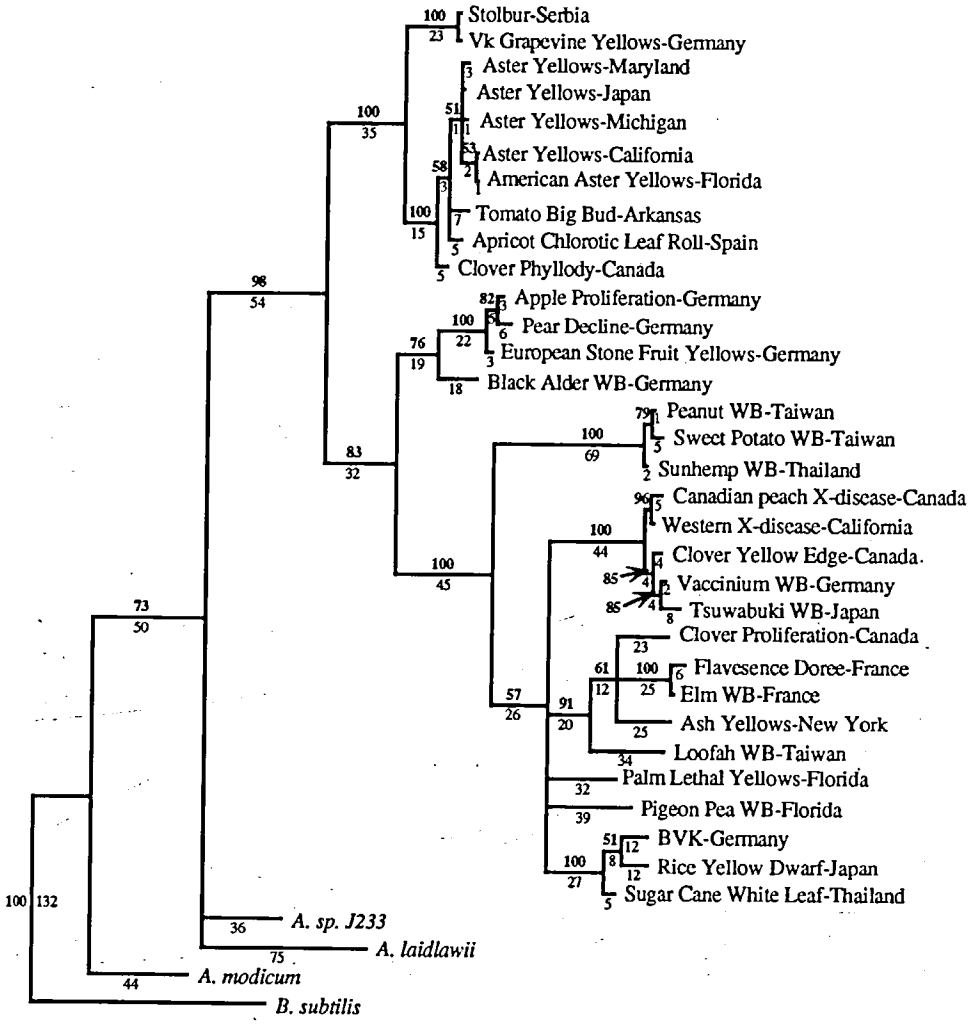


Fig. 3. Phylogram of 16S rRNA sequences generated using a PAUP bootstrap analysis (Swofford, 1993). Numbers in bold above the branches indicate the bootstrap values, whereas numbers below the branches indicate branch length. *B. subtilis* was used as an outgroup.

16S rRNA sequences that were available at the time this chapter was written. A representative phylogenetic tree based on the full-length phytoplasma 16S rRNA sequences that were available at the time this chapter was written is presented in Fig. 3, whereas Fig. 4 shows the relationships of phytoplasmas based on SR sequences.

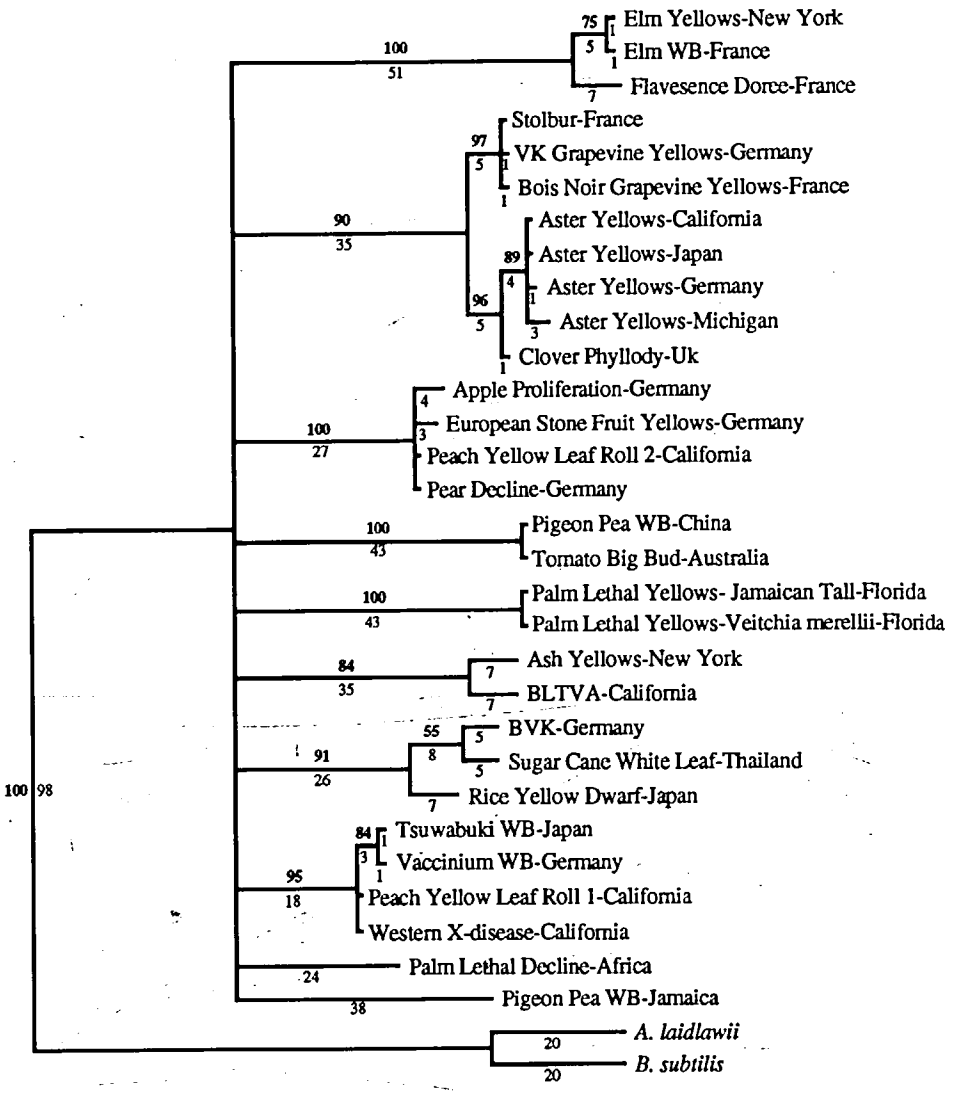


Fig. 4. Phylogram of 16S/23S spacer region sequences generated using a PAUP bootstrap analysis (Swofford, 1993). Numbers in bold above the branches indicate the bootstrap values, whereas numbers below the branches indicate branch length. *A. laidlawii* and *B. subtilis* were used as outgroups.

Discussion

There are advantages and disadvant
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However, sequence analysis of either
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Kirkpatrick, B. C., Smart, C. D., Gardner, S. L.
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Kuske, C. R., and Kirkpatrick, B. C. (1992). Ph
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Lee, I.-M., Hammond, R. W., Davis, R. E., and
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Lim, P.-O., and Sears, B. B. (1989). 16S rRNA
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Olsen, G. J., Woese, C. R., and Overbeek, R. (19
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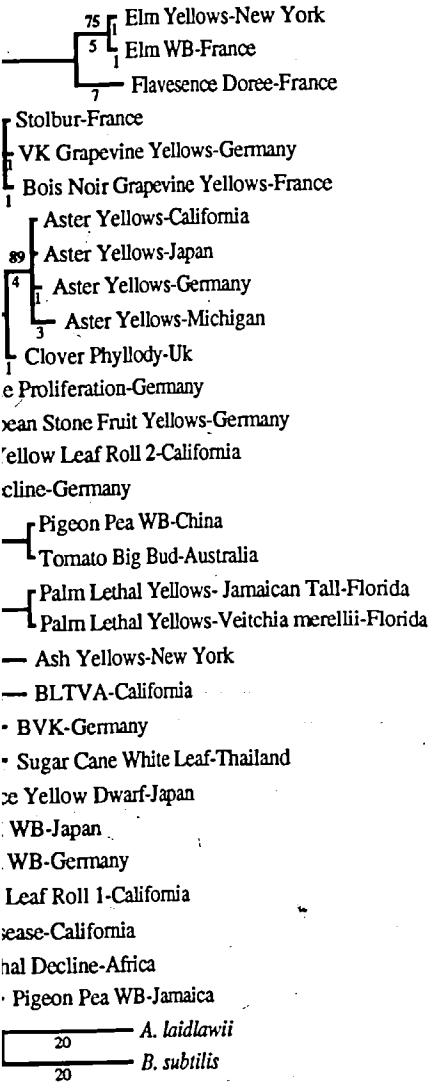

sequences generated using a PAUP bootstrap
ve the branches indicate the bootstrap values,
branch length. *A. laidlawii* and *B. subtilis* were

Discussion

There are advantages and disadvantages associated with both RFLP and sequence analysis of rDNA for classifying phytoplasmas. As previously discussed, a more accurate assessment of the phylogenetic relationship of an unknown phytoplasma strain is obtained from rDNA sequences than from RFLP analysis. However, sequence analysis of either the SR or the 16S rRNA gene requires considerably more time and effort. RFLP analysis of the PCR-amplified 16S rRNA gene is a rapid method to assess the potential affinity of an unknown phytoplasma. However, the total amount of information analyzed by this approach is less than that obtained by sequence analysis. Consequently, RFLP analysis does not provide a detailed picture of the relationships between phytoplasmas within a particular clade.

Similar types of advantages and disadvantages are associated with sequencing either the full-length 16S rRNA gene or the 16/23S spacer region. The SR is comparatively easy to sequence and a large number of phytoplasma SR sequences have been determined (Kirkpatrick *et al.*, 1994; manuscript in preparation). However, the larger size of the 16S rRNA gene allows the most definitive comparisons to be made, and the large database that is now available for both culturable and nonculturable prokaryotes continues to make this marker the most phylogenetically useful gene to analyze.

References

Gunderson, D. E., Lee, I.-M., Rehner, S. A., Davis, R. E., and Kingsbury, D. T. (1994). Phylogeny of mycoplasmalike organisms (phytoplasmas): A basis for their classification. *J. Bacteriol.* **176,** 5244–5254.

Kirkpatrick, B. C., Smart, C. D., Gardner, S. L., Gao, J.-L., Ahrens, U., Maurer, R., Schneider, B., Lorenz, K.-H., Seemuller, E., Harrison, N. A., Namba, S., and Daire, X. (1994). Phylogenetic relationships of plant pathogenic MLOs established by 16/23S rDNA spacer sequences. *IOM Lett.* **3,** 228–229.

Kuske, C. R., and Kirkpatrick, B. C. (1992). Phylogenetic relationships between the western aster yellows mycoplasmalike organisms and other prokaryotes established by 16S rRNA gene sequence. *Int. J. Syst. Bacteriol.* **42,** 226–233.

Lee, I.-M., Hammond, R. W., Davis, R. E., and Gundersen, D. E. (1993). Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasmalike organisms. *Phytopathology* **83,** 834–842.

Lim, P.-O., and Sears, B. B. (1989). 16S rRNA sequence indicates plant pathogenic mycoplasmalike organisms are evolutionarily distant from animal mycoplasmas. *J. Bacteriol.* **171,** 1233–1235.

Namba, S., Oyaizu, H., Kato, S., Iwanami, S., and Tsuchizaki, T. (1993). Phylogenetic diversity of phytopathogenic mycoplasmalike organisms. *Int. J. Syst. Bacteriol.* **43,** 461–467.

Olsen, G. J., Woese, C. R., and Overbeek, R. (1994). The winds of (evolutionary) change: Breathing new life into microbiology. *J. Bacteriol.* **176,** 1–6.

Schneider, B., Ahrens, U., Kirkpatrick, B. C., and Seemuller, E. (1993). Classification of mycoplasma-like organisms using restriction-site analysis of PCR-amplified 16S rDNA. *J. Gen. Microbiol.* **139,** 519–527.

Seemuller, E., Schneider, B., Maurer, R., Ahrens, U., Daire, X., Kison, H., Lorenz, K.-H., Firrao, G., Avinent, L., Sears, B. B., and Stackebrandt, E. (1994). Phylogenetic classification of phytopathogenic mollicutes by sequence analysis of 16S ribosomal DNA. *Int. J. Syst. Bacteriol.* **44,** 440–446.

Swofford, D. L. (1993). PAUP: Phylogenetic analysis using parsimony, version 3.1.1. Computer program distributed by the Illinois Natural History Survey, Champaign, IL.

Weisberg, W. G., Tully, J. G., Rose, D. L., Petzel, J. P., Oyaizu, H., Mandelco, L., Sechrest, J., Lawerence, T. G., Van Etten, J., Maniloff, J., and Woese, C. R. (1989). A phylogenetic analysis of the mycoplasmas: Basis for their classification. *J. Bacteriol.* **171,** 6455–6467.

DETERMINATION O AND POLYOXYETHY GROWTH REQUIRE OF MOLLICUTES

Joseph G. Tully

General Introduction

Determination of cholesterol or ste important element in the classificatio licutes. Although recent reports have vary within certain phylogenetic group 1993; Tully *et al.*, 1993), tests to meas to polyoxyethylene sorbitan (Tween 8 niques in the minimum standards for d (see Chapter E2, this volume; Internati ogy Subcommittee, 1995). Cholestero quantitative comparison of growth o various supplements of fatty acids, albu 1–20 μg/ml) of solubilized cholesterol various organisms assigned to the Ento Anaeroplasmataceae show minimal or but enhanced growth in the presence addition, a newly described group of growth requirement for serum or cho serum-free media containing low conc

Molecular and Diagnostic Procedures in Mycoplasmology, Vol.