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PHYTOPLASMA: AN INTRODUCTION AND CLASSIFICATION WITH RECENT MOLECULAR TOOLS

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ABSTRACT

Since their discovery in 1967 as 'mycoplasma-like organisms', the phytoplasmas have quickly become established as a unique group of plant pathogens. Diseases, frequently called 'yellows', have been known since the late 1800s; originally thought to be associated with viruses, many are now known to be caused by phytoplasmas. During the 1970s, research centered on diagnosis using symptoms and electron microscopy to visualize the phytoplasmas in the phloem sieve cells of their hosts, transmission by insect vector and studies on the spread of the diseases they caused. The biology and taxonomy of these obligate pathogens were still shrouded in mystery. It was the advent of the molecular biological revolution in the 1980s that saw the introduction of techniques such as nucleic acid purification, DNA hybridization and the polymerase chain reaction, which with the secrets of these fastidious bacteria begin to emerge. In the 1990s the term phytoplasma had been proposed, and by 2004 a distinct taxonomic group, '*Candidatus* Phytoplasma', was defined. The evolution of molecular techniques has led to more information and, paradoxically, less clarity in grouping different phytoplasma 'taxa'.

Keywords: Phytoplasmas, PCR, RFLP

INTRODUCTION

Phytoplasmas, previously referred to as mycoplasma like organisms (MLOs) are small wall less prokaryotes that descended from an ancestral low G+C gram positive bacterium, possibly a *Clostridium* like member of *Lactobacillus* lineage (**Woese,**

1987). Earlier all such diseases were known to be caused by viruses until phytoplasmas were first time observed by Japanese workers **(Doi *et al.,* 1967)** as pleomorphic cells in ultra-thin sections of leaves of mulberry infected with dwarf disease. Phytoplasmas are single celled (round to filamentous, 200-800 nm) wall less obligate parasites that can't be grown in cell free culture *in-vitro* (**Lee *et al.,* 1986).** Phytoplasmas are sensitive to tetracycline but resistant to penicillin **(Ishii *et al.,* 1967)** and transmitted by phloem feeding insects; leafhoppers, planthoppers, psyllids and *Cuscuta*. Phytoplasmas are causing a number of economically important plant diseases world-wide.

Along with Spiroplasmas, Acholeplasmas and other cell wall less bacteria, phytoplasmas are classified in the class *Mollicutes.* To date no phytoplasma culture has been established *in-vitro,* so classification and differentiation of phytoplasmas by means of biophysical and biochemical based criteria that are routinely used for culturable micro-organisms have been impossible. Earlier attempts to differentiation and classification based on biological properties were not found to be reliable and accurate. In the 1980s and early 1990s the employment of serological and nucleic acid based techniques revealed new insights into diversity and genetic interrelationships of phytoplasmas. In particular, based on restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR)-amplified 16S rRNA, Lee and colleagues structured first comprehensive phytoplasma classification scheme **(Lee *et al.,* 1998)**. As per the taxonomic notes by Phytoplasma/Spiroplasma Working Team of International Research Program of Comparative Mycoplasmology (IRPCM) and International Committee on Systematic Bacteriology (ICSB) - Subcommittee on the Taxonomy of *Mollicutes*, phytoplasma are to be given the tentative names of *Candidatus* species and there they are to described based on molecular, biological as well as phytopathological properties.

Over the last few years numerous and diverse phytoplasmas have been discovered at an increasingly rapid pace in emerging diseases world-wide. These developments have raised expectations that the number of 16S rRNA RFLP groups (16Sr groups) and subgroups could rise considerably, so various attempts to expand phytoplasma classification are being made. As it is necessary to study the properties of plant pathogens so as to formulate efficient disease management strategies and it is not possible to study each and every isolate in detail so a stable and reliable classification system is needed and it has been accepted to

have a classification system based on molecular properties although it is hoped to have new insights to phytoplasmas as the research in subject will advance. Thus studies on phytoplasmas in general and classification systems in particular are in transition stage and need further more extensive research to have an acceptable classification system.

History

After the discovery by **Doi *et al.,*** in **1967** causal agents of yellow disease were named as Mycoplasma Like Organisms (MLOs) due to their morphological resemblance with Mycoplasmas, but later on it was realized that these wall less prokaryotes infecting plants are different from Mycoplasmas in many characteristics, so the trival name "Phytoplasma" was adopted in 10th congress of International Organization of **Mycoplasmology (1994**) to refer these organisms. Thereafter it was found that various diseases which were earlier known to be caused by viruses were actually caused by phytoplasmas. Earlier phytoplasmas were detected by the presence of pleomorphic cells in the sieve tube elements of infected plants when ultra thin sections were stained with DAPI (4-6-diamino-2-phenylindole), which binds with the nucleic acid present in cytoplasm and gives florescence under UV light.

Then detection based on serological assays was tried and polyclonal and monoclonal antibodies were employed for the detection. But the difficulty in obtaining pure phytoplasmas from infected plants limited the popularization of serological assays.

International Committee on Systematic Bacteriology (ICSB) - Subcommittee on the Taxonomy of *Mollicutes* has been assigned the work of comprehensive studies on *Mollicutes* and for specific studies on plants infecting *Mollicutes*, Phytoplasma/Spiroplasma Working Team of International Research Program of Comparative Mycoplasmology (IRPCM) has been formulated. More recently with the advent of molecular era which employs DNA hybridization assays and PCR based assays became popular for detection and classification purposes. As 16S rRNA gene has widely been used for classification and phylogenetic studies of prokaryotes so same has been tried for classification of phytoplasmas. Various less conserved regions like 23S rRNA gene, 16S-23S spacer, transcription elongation factor (*tuf*) have also been tried by various workers for finer classification.

Phytoplasmas as Novel Plant Pathogens

Phytoplasmas are known to infect various fruit plants, trees, ornamental plants and herbacious annuals. Disease caused by phytoplasma were reported quite earlier

than the discovery of their causal agents. Disease like clover phyllody (**Merrett, 1666),** peach yellow **(Smith, 1888),** aster yellow on China aster **(1902**), has been reported quite earlier in literatature but the first etiology was studied for mulberry dwarf disease by **Doi *et al.*, 1967**. Till now greater than 300 plant diseases in hundreds of plant genera are reported to be caused by phytoplasmas.

Characteristic Symptoms of Phytoplasma Diseases:

Symptoms Due to Phytoplasma Infection Includes:

- **Virescence:** Development of green flowers, loss of normal flower pigment.
- **Phyllody:** development of floral parts to leafy structures.
- **Little Leaf:** small leaves.
- **Sterility of Flowers**
- **Witches' broom:** Proliferation of axillary shoots.
- **Slender shoots.**
- **Stunting and leaf curling.**
- **Generalized decline, Bunchy growth etc.**

Internal symptoms include:

- Phloem necrosis
- Excess formation of phloem tissues
- Swollen veins

Economically Most Important Plant Diseases World wide:

- Aster yellows in Carrot and Onion
- Apple proliferation
- Coconut lethal yellowing
- Peach X disease

Aster yellow group contributes major economical losses to many vegetable crops and ornamentals. As far as India is concerned various economically important disease are caused by phytoplasmas like sandal spike, coconut root wilt, phyllody of various crops *etc*. as listed in **Table 1**.

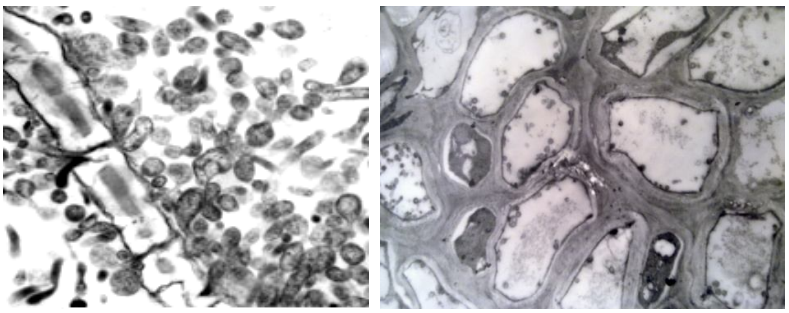

Figure 1: Phytoplasmas Seen in Sieve Tube Elements of Plants Under Electron Microscope

Table 1: Important Phytoplasma Diseases in India

Disease	Host	Area	First Report of etiology
Little leaf	Brinjal Periwinkle	All India Lukhnow	Varma *et al*. (1969*)* Rao *et al*. (1983)
X disease	Peach	NE region	Ahlawat & Chenulu (1979)
Bushy Stunt	Brinjal	New Delhi	Mitra & Chakraborty (1988)
Phyllody	Bottle gourd & other gourd Black pepper Sesame	Banglore Banglore Karela All India	Sastry & Singh (1981) Bhat *et al*. (2006) Sahambi (1970)
Witches' broom	Acid Lime Winged bean Sunhemp	MH, AP	Ghosh *et al*. (1999) Singh (1991) Sharma *et al*. (1990)
Rubbery wood	Citrus	Darjeeling	Ahlawat & Chenula (1985)
Root wilt	Coconut	Kerala	Solomon *et al*. (1983)
Sandal spike	Sandal	Kerala,Kr	Varma *et al*. (1969*)*
GSD	Sugarcane	All India	Rishi *et al*. (1973)
Yellow dwarf	Rice	All India	Raychaudhri *et al*. (1967)

Phytoplasma Classification

"*After a time the growth of and accumulation of specimens or phenomena forces people to try to classify*" - **Piere 1995**.

Classification is the arrangement of biological entities to different taxonomic categories based on their similarities and relationship.

There are certain hurdles to the definite description and classification of phytoplasmas:

- **Obligate parasitic habit:** As phytoplasmas can't be grown *in-vitro* so it is very difficult to study their phenotypic properties.
- **Structural fragility:** Due to lack of cell wall and pleomorphic nature of cells they are difficult to isolate in pure forms and moreover they could pass through membranes.
- **Presence in low numbers in infected plants**: Phytoplasmas are present in very low concentration in their respective host.
- **Intimate association with host tissues:** Phytoplasmas are intracellular pathogens and intimately associated with the hosts.

Development of Phytoplasma Classification Systems:

Phytoplasma classification system developments, from beginning to now can be studied under the following headings:

1. Based on biological properties (1970s)
2. Based on serological properties (1980s)
3. Based on molecular properties (1990s onward)

Attempts to Phytoplasma Classification:

Based on Biological Properties:

In earlier attempts to classification biological properties has been utilized by various workers for phytoplasma classification like:

- **Symptomatology:** Symptoms induced by phytoplasmas on their host plants has been utilized for classification.
- **Host Range:** Depending on the host species which phytoplasmas infect eg. Aster yellow, Clover phyllody.
- **Transmission by insect vectors**: Based on which vector species is involved in the transmission of phytoplasma.

Biological Era

Based on symptoms phytoplasma has been classified to various groups:

Grunewald *et al.* (1977) classified phytoplasmas in to five major groups: (i) Aster yellows (ii) Stolbur (iii) Witches' broom (iv) Decline (v) Phyllody

Kirkpatrick *et al.*, 1992, classified phytoplasmas in to three major groups based on symptoms:

i. Decline agents: Phytoplasmas causing decline in overall growth of trees.

ii. Proliferation agents: Causing phyllody symptoms and slender shoots etc.

iii. Virescence agents: Causing abnormalities on inflorescence.

Chyokowski and Sinha, 1989, classified phytoplasmas in to two major groups:

i. Those phytoplasmas causing mutually exclusive floral symptoms.

ii. Those causing reduced flower size and colour along with other symptoms, both produced on experimental host periwinkle (*Catharanthus roseus*).

However it was realized that classification based on biological properties is not reliable as same phytoplasma strain may induce different symptoms in different hosts and different phytoplasma strains may share a common vector(s) or cause diseases characterized by similar symptoms, thus this approach could not provide an accurate means for phytoplasma classification.

Serological Era

Various serological based methods have been utilized for phytoplasma studies. Serolgical methods are mostly used for phytoplasma detection and less commonly for classification. Phytoplasma enrichment extraction procedure has been used to get good quantity of phytoplasmas from infected host plants. Monoclonal antibodies were produced and utilized for strain identification **(Chen *et al.*, 1988)**. Polyclonal antibodies were utilized for phytoplasmas detection **(Kirkpatrick *et al.*, 1992)**.

But it was realized that serology is difficult to utilize in phytoplasma studies because of the reasons:

- It is difficult to obtain pure phytoplasmas so difficult to produce antibodies.
- Phytoplasmas are present at very low concentration in plant tissues and intimately associated with plants.
- There are every chances of non specific reactions.

Molecular Era

With the advent of molecular techniques phytoplasma detection and classification improved a lot and employment of molecular techniques gave new insights to phytoplasma properties and characteristics.

Classification Based on DNA-DNA Hybridization Assays

In 1ate 1980s and early 1990s dot and southern hybridizations using cloned phytoplasma DNA probes permitted studies of genetic interrelationships among phytoplasmas, resulting in the recognition of several distinct phytoplasma groups (genomic strain clusters) and subgroups (subclusters). Differentiation of strains within a given strain cluster was easily achieved by southern hybridization. **Lee *et al.*, 1992**, used a substantial number of cloned phytoplasma DNA probes to establish the first genotype based – classification scheme for differentiation of strains in the aster yellows phytoplasma cluster. Their results revealed that DNA based hybridization could differentiate closely related strains.

Lee & Davis, 1992, cloned DNA fragments from known phytoplasmas and used for hybridization assays. It was found that each Strain Cluster consists of strains with extensive sequence homology as shown in **Table 2**.

Difficulties with DNA - Hybridization Assays

- DNA fragments were cloned only from limited numbers of Phytoplasmas.
- Difficulty in obtaining desired concentration of phytoplasma strains from infected hosts.
- Standardized DNA probes for general detection and identification were not available.

Keeping these difficulties in view scientists opted for PCR base assays for phytoplasma classification.

Polymerase Chain Reaction Based Assays for Phytoplasma Classification

PCR based assays provide a much more sensitive means than serological and DNA-DNA hybridization assays for phytoplasma detection and classification. Initially, PCR primers were designed based on sequences of cloned phytoplasma DNA fragments and

were used for identification of specific phytoplasmas. Various workers designed phytoplasma universal (generic) or phytoplasma group specific oligo-nucleotide primers based on highly conserved 16S rRNA gene. Universal and phytoplasma group-specific primers were also developed based on 16S-23S inter spacer region sequences or conserved ribosomal protein gene and elongation factor EF-Tu (*tuf*) gene sequences.

Recent Molecular Tools for Phytoplasma Classification

16S rRNA gene: It is most conserved region among prokaryotes and used for phytoplasma group level classification. Universal primers - P1/P7 and R16F2n/R16R2 were used for 16S rRNA gene amplification **(Figure 2)**.

Less conserved region: These regions are used for finer subgroup classification:

- **23S rRNA**
- **16S-23S spacer:** Inter-genic spacer between 16S and 23S rRNA gene
- **Ribosomal protein gene operon (*rpl*22, *rps*3):** For amplification of rp gene primers-rpF1, rpR1are being used.
- ***Tuf* elongation factor**

Table 2: Strain Clusters Based on DNA Hybridization

S. No.	Strain Cluster	Reference
1	Little leaf disease of periwinkle-MLO	Davis *et al.* (1990)
2	Ash yellows-MLO	Davis *et al.* (1991)
3	Clover proliferation-MLO	Lee *et al.* (1992)
4	Aster yellows-MLO	Lee *et al.* (1992)
5	Canadian peach X disease , Western X disease, Clover yellow edge	Lee *et al.* (1992)
6	Italian periwinkle virescence	Davis *et al.* (1992)

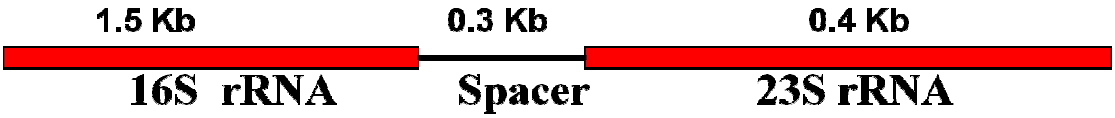

Figure 2: 16 S rRNA and 23 S rRNA Gene Showing 16S rRNA gene, 23S rRNA Gene Separated by Inter-Genic Spacer

When various gene region sequence similarity was compared it was found that phytoplasmas share a sequence similarity of 88-99% for 16S rRNA with in Phytoplasma strain cluster and a similarity of 87.0-88.5% with their closest known relatives *Acholeplasma*. Whereas sequence similarity for ribosomal protein gene with in

phytoplasma strain cluster is 60-79% and 50-57% with *Acholeplasma* showing that 16S rRNA region is most conserved and could be utilized for broad level classification while for finer classification and differentiation to subgroup level less conserved regions like ribosomal protein (rp) should be utilized.

Phytoplasma Classification based on RFLP of PCR amplified 16S rDNA Schneider *et al.*, 1992

16S rRNA gene amplification procedure depends on presence of restriction site of *Bcl*I in 16S rDNA of chloroplast but not in that of phytoplasmas. In this procedure extracted DNA was first digested with *Bcl*I and then amplified with primers from conserved regions which allows the amplification of only phytoplasma 16S rDNA and not of chloroplasts.

In Schneider's experiment a total of 52 isolates were taken for study comprising of isolates collected from various regions and some sequences like Oenothera (OAY) and western aster yellow were taken for comparison. Some other prokaryotes like *Agrobacterim tumaefaciens, Erwinia amylovora, Xanthomonas campestris, E. coli, Spiroplasma citri* were also included in study. For amplification five different primers from conserved region of 16S rRNA gene were used. Pair fD1 and rP1 primed proximal to the 5' and 3' termini allowing amplification of full 16S rDNA region. DNA from healthy and diseased plants was obtained using phytoplasma enrichment procedure (Ahrens and Seemuller). After 30 amplification cycles electrophoresis was done on 1% agrose gel. From the band containing desired 16S rDNA, some material was removed with hypodermic syringe and amplified again. Purity of final product was examined by digesting again with *Bcl*1 digestion followed by gel electrophoresis. Then final product was digested with *Alu*1, *Rsa*1, *EcoR*1. Amplified DNA was cloned and sequenced using kit method. Sequence comparison was done with multiple alignment CLUSTAL-X programme.

Gel electrophoresis resolved one fragment approximately 1500 Kb representing 16S rRNA gene of phytoplasmas. 52 isolates examined were grouped according to presence of *Alu*1 and *Rsa*1 restriction sites. Restriction digestion of amplified 16S rDNA with *Alu*1 revealed different profiles among phytoplasma isolates which was used to depict phytoplasmas to seven major groups.

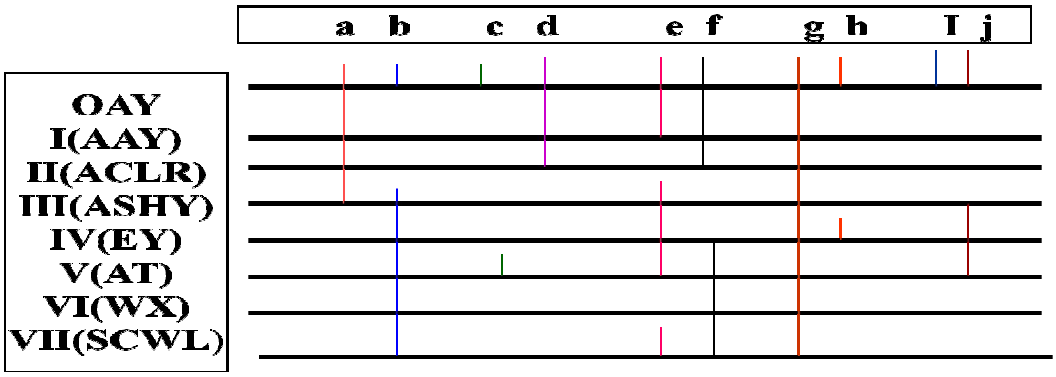

Figure 3: *Alu*I restriction map of 16S rDNA depicting the seven (I-VII) restriction profiles. Where OAY: Oenothera Aster Yellows, AAY: American Aster Yellow, ACLR: Apricot Chlorotic Leaf Roll, ASHY: Ash Yellow, EY: Elm Yellow, AT: Apple Proliferation, WX: Western X disease, SCWL: Sugarcane White Leaf

For comparison of restriction sites Oenothera Aster Yellow (OAY) sequence was taken as standard. Group 1 with representative strain AAY was largest with 31 isolates, mostly from herbaceous dicots with few woody host plants.

All isolates from group 1 showed five restriction sites at positions a, d, e, f and g. Group II to VI consisted of isolates mostly from woody plants. Group II had no 'e' site while group III not had 'd' and 'f' sites but additional sites 'b' and 'I'. Group IV had not 'd' and 'f' sites but additional sites 'h' and 'j'. Group V consist of additional site 'c'. Group VI had only three sites 'b', 'f' and 'g'. Group VII was found somewhat related to group V. RsaI restriction analysis discovered identical grouping as recovered by *Alu*I. All isolates show unique restriction site *Eco*RI in 16S rDNA at position 669 of OAY-MLO.

It was successfully demonstrated that even though phytoplasmas can't be cultivated *in-vitro*, the 16S rDNA of phytoplasmas can be readily obtained by PCR amplification and utilized for taxonomic studies.

On the basis of restriction pattern analysis phytoplasmas in this study were divided to seven groups (about 60% of them belongs to group I) in which phytoplasmas from all symptom groups (aster yellows, clover phyllody, periwinkle virescence, stolbur) were represented, showing that 16S rRNA classification is much finer than symptoms based classification. DNA hybridization studies also supported the same. When sequence similarity of different phytoplasma isolates was compared it was found that isolates from same group shared 97.8 to 99.5% similarity while isolates from different groups shared a similarity of 89.6 to 92%.

Comprehensive Classification Based on 16S rRNA Gene Lee *et al.*, 1993

This study provided PCR based classification using universal pair of primers

(Ahrens and Seemuller, 1992) and RFLP of amplified product with standard restriction enzymes. 40 phytoplasma strains were taken for study. Three set of primer pairs were evaluated for amplification and primer pair R16F2/R2 was standardized. Total nucleic acid was extracted and 35 PCR amplification cycles were run.

RFLP Analysis

In this particular study of Lee, 3-5 micro litre of amplified PCR product was digested with 15 restriction enzymes; *Mse*I, *Alu*I, *Hpa*I, *Hpa*II, *Sau*IIIA, *Rsa*I, *Hinf*I, *Taq*I, *Hha*I, *Hae*III, *Kpn*I, *Dra*I, *EcoR*I, *Tha*I, *EcoR*II. Restriction product was then separated by gel electrophoresis on 5% polyacrylamide gel. RFLP pattern (sum of results analysed by restriction enzymes) of 40 phytoplasma strains were compared and analysed by Nei and Lei method (calculation of similarity coefficient, F) given by **Nei and Lei, 1979**. Similarity coefficient (F) = 2Nxy/ Nx+Ny where Nx and Ny are the number of fragments (resulting from digestion by 15 restriction enzymes) in strain x and y respectively, and Nxy is the number of fragments shared by the two strains.

Of the three primer pairs R16F0/R0 and R16F0/R2 gave amplification of 16S rDNA sequences from DNA samples of healthy as well as from infected plant samples. While primer pair R16F2/R2 gave 1.2 Kb amplification of infected samples only. Referential pattern of RFLP for comparison of new phytoplasma strain was constructed as shown in **Table 3**.

Based on similarity coefficient threshold value of greater than 0.9 for same group all phytoplasma strains were divided in to 9 major 16Sr groups and 14 subgroups. Out of which 16Sr group I was found to be largest one with 5 subgroups and 16SrIII was having 2 subgroups. A comparison of 16Sr groups with previous strain clusters identified based on DNA hybridization assays was done and it was found that grouping based on RFLP of 16S rRNA gene was consistent with previous strain clusters as shown in **Table 4**.

Table 3: Reference RFLP Pattern of 9 Groups for Comparison of New Phytoplasma Strain

Group	*MseI*	*AluI*	*HpaI*	*HpaII*	*Sau3A*	*RsaI*	*HinfI*	*TaqI*	*HhaI*	*Hae III*	*KpnI*	*DraI*	*EcoRI*	*ThaI*	*EcoRII*
I-A	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
I-B	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1
I-C	2	2	1	1	1	1	1	1	1	2	1	1	1	1	1
I-D	3	1	1	1	1	1	1	1	2	1	1	1	1	1	1
I-E	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1

II	4	3	1	3	2	2	2	2	3	2	2	1	1	1	1
IIIA	5	4	1	4	3	2	2	1	1	3	2	1	1	2	2
III-B	6	4	1	5	3	2	2	1	1	3	2	1	1	2	2
IV	7	5	1	4	3	3	3	1	1	3	2	1	1	2	2
V	8	6	1	4	3	4	2	1	3	4	2	1	1	2	3
VI	9	7	1	4	3	5	2	1	4	4	2	1	1	2	3
VII	10	8	1	4	3	5	2	3	5	3	2	1	1	2	3
VIII	8	4	1	4	3	5	2	1	4	4	2	1	1	1	3
IX	11	9	1	4	3	6	4	4	6	5	2	2	1	1	3

Table 4: Comparison of 16Sr Groups with Previous Strain Clusters

Lee *et al*. (1993)-16Sr groups	Lee & Davis (1992)-Strain Clusters
16SrI(5 subgroups)	AY-MLO
16SrII	Peach X disease -MLO
16SrV	EY -MLO
16SrVI	CP-MLO
16SrVII	Ash Y -MLO

Identification of Key Enzymes for Group and Subgroup Classification:

Key enzymes were determined for group and subgroup classification and it was found that *Mse*I and *Alu*I were sufficient for classification of a new strains to 16SrI, while restriction profile from *Mse*I, *Alu*I, *Hpa*II &*Hha*I was needed for further classification to subgroup.

Again to verify the classification system, authors took two new unclassified strains Blueberry stunt (BBS) and Hydrangia phyllody (HyPhI) and studied the restriction pattern with 15 restriction enzymes and were able to classify them to 16SrI-B and 16SrI-E respectively.

Revised Classification Based on RFLP of 16S rRNA and Ribosomal Protein Gene Sequence

In this study **Lee *et al.*, 1998,** selected 34 phytoplasma strains consisting of previously classified representative strains from each group and new unclassified strain. Nucleic acid was extracted from 34 strains by phytoplasma enrichment procedure and nested PCR was performed by using nested pair of primers. 17 standard restriction enzymes were used for calculation of similarity coefficient and for classification.

Based on similarity coefficient value (threshold value of greater than 0.9 for same group), 34 phytoplasma strains were classified to 14 major 16Sr groups and 32 subgroups. Then 16S rDNA sequence data of the other phytoplasmas was also included and thus a total of 14 major 16Sr groups and 41 subgroups (16Srp) were proposed. By combined RFLP analysis of 16S rRNA and ribosomal protein gene protein gene

sequence a total of 46 subgroups were recognized. When combined RFLP analysis of 16S rRNA gene and ribosomal protein gene was done it was possible to have more finer classification to subgroups by analysis of restriction enzymes.

It provided a valid classification system consistent with hybridization and serological assays. Approach for classification was based on:

- **16S r Groups:** 16S rRNA gene sequence.
- **Subgroups:** 16S r RNA gene & rp gene cluster **(Table 4a)**.

  When 16S rRNA gene sequence homology was studied it was found that two distinct 16S rRNA groups shared sequence homology of 88-94%, while two subgroups with in a group shared a sequence homology of 95-98%.

Table 4a: Comparison 16Sr Subgroups from Group 16Sr1, in Brackets Subgroups Obtained from Ribosomal Protein RFLP are shown

Strain	16S r-rp subgp.	Strain	16S r-rp subgp
Tomato big bud BB	16SrI-A (rp-A)	Maize bushy stunt MBS	16SrI-B (rp-L)
New Jersey AY NJAY	16SrI-A (rp-A)	Clover phyllody CPh	16SrI-C (rp-C)
Periwinkle little leaf CNI	16SrI-A (rp-A)	Strawberry green petal SGP	16SrI-C (rp-C)
Oklahoma AY OKAYI	16SrI-A (rp-A)	Annulus phyllody RPh	16SrI-C (rp-C)
Maryland aster yellow AYI	16SrI-B (rp-B)	Paulownia WB PaWB	16SrI-D (rp-D)
Dwarf aster yellow DAY	16SrI-B (rp-B)	Blueberry stunt BBSI	16SrI-E (rp-E)
Hydrangea phyllody HyPH	16SrI-B (rp-K)	Grey dogwood WB GDI	16SrI (rp-M)
Ipomoea WB IOB	16SrI-B (rp-F)		

***Candidatus* Phytoplasma Approach**

Phytoplasma/Spiroplasma Working Team **IRPCM, 2000,** Various taxonomic notes were proposed by various workers for phytoplasma classification so as to have tentative genus and species name for phytoplasma also like other prokaryotes.

Table 5: Taxonomic Notes

Taxonomic Notes	Reference
Major part of gene to be sequenced (1000bp from 16S rRNA) for taxonomy.	Murray *et al*., 1990
Candidatus (*L. Candidatus*, a candidate to indicate that assignment is provisional) and must include: ▪ Sequence (16S rRNA) ▪ Identification of morphotype with probes from characteristic sequence	Murray and Schleifer, 1994
Organisms with less than 97% sequence homology of 16S rRNA will not have more than 60-70% reassociation	Stackebrandt and Goebel, 1994

It was accepted by Phytoplasma/Spiroplasma Working Team **IRPCM, 2000,** that all phytoplasmas must be given the rank of *Candidatus* phytoplasma equivalent to genus and in each 16Sr group atleast one *Candidatus* phytoplasma species must be described.

Description of *Candidatus* Phytoplasma

Table 6: Characteristics of Genus *Candidatus* Phytoplasma

Character	Description	Refrence
Morphology	**Single unit membrane, pleomorphic**	**Doi *et al.*, 1967**
Habitat	**Phloem sieve, gut, haemolymph of sap-sucking insects**	**Tsai *et al.*, 1979**
Ribosomal RNA	**Two rRNA operons & a spacer 16S-23S rRNA genes**	**Kuske & Kirkpatrick, 1992**
Antibiotic sensitivity	**Tetracycline**	**Ishii *et al.*, 1967**
DNA base composition	**G+C : 23-29%**	**Kollar & Seemuller, 1989**
Chromosomal Size	**530-1350 bp**	**Neimark & Kirkpatrick, 1993**
Codon usage	**UGA- stop codon, not for tryptophan**	**Lims & Sears, 1991**
Sterol in membrane	**Non sterol requiring**	**Lim *et al.*, 1992**

Description of New *Candidatus* Phytoplasma Species:

In order to prevent nomenclatural confusion that may arise from the description of poorly differentiated novel taxa, the Phytoplasma/Spiroplasma Working Team **IRPCM, 2000,** suggested rules for the description of organisms as novel taxa with in *Ca*. phytoplasma as follows:

1. Single, unique 16S rRNA gene sequence (>1200bp) from the 'reference strain'
2. A strain should be called as novel *Ca*. Phytoplasma sp if it has <97.5% sequence similarity with previously defined *Ca*. Phytoplasma species.
3. Even if it has >97.5% similarity but ecologically separated population *i.e*.
    a. Transmitted by different vectors,
    b. Have different natural plant host/ different response on same host,
    c. Significant molecular diversity (DNA probe hybridization, serology, PCR assay).
4. No rank of subspecies is to be given for phytoplasmas.
5. Description of new species submitted to *International Journal of Systematic and Evolutionary Microbiology (IJSEM).*

6. Reference strain should be made available to scientists.

7. Abbreviation of *Candidatus* is *Ca*.

Table 7: Described *Ca*. Phytoplasma Species in Different Groups

Phylogenetic Group	*Candidatus* Phytoplasma sp.	Reference
Aster yellows (16SrI)	*Ca*. Phytoplasma asteris	Lee *et al*., 2004a
Peanut witches'-broom (16SrII)	*Ca*. Phytoplasma aurantifolia	Zreik *et al.*, 1995
X-disease(16SrIII)	*Ca*. Phytoplasma pruni	
Coconut lethal yellowing (16SrIV)	*Ca*. Phytoplasma palmae *Ca*. Phytoplasma cocostanzaniae *Ca*. Phytoplasma castaneae	
Elm yellows (16SrV)	*Ca*. Phytoplasma ziziphi *Ca*. Phytoplasma vitis *Ca*. Phytoplasma ulmi	Jung *et al*., 2003a Lee *et al*., 2004b
Clover proliferation (16SrVI)	*Ca*. Phytoplasma trifolii	Hiruki & Wang, 2004
Ash yellows(16SrVII)	*Ca*. Phytoplasma fraxini	Griffith *et al*., 1999
Loofah witches'-broom (16SrVIII)	*Ca*. Phytoplasma luffae	
Pigeon pea witches'-broom (16SrIX)	*Ca*. Phytoplasma phoenicum	Verdin *et al*., 2003
Apple proliferation (16SrX)	*Ca*. Phytoplasma mali *Ca*. Phytoplasma pyri *Ca*. Phytoplasma prunorum	Seemuller & Schneider, 2004
Rice yellow dwarf (16SrXI)	*Ca*. Phytoplasma oryzae	Jung *et al*., 2003b
Stolbur (16SrXII)	*Ca*. Phytoplasma australiense *Ca*. Phytoplasma japonicum	Davis *et al.*, 1997 Sawayanagi *et al*., 1999
Mexican periwinkle virescence (16SrXIII)	No name suggested	
BGWL (16SrXIV)	*Ca*. Phytoplasma cynodontis	Marcone *et al*., 2004
Hibiscus witches'-broom (XV)	*Ca*. Phytoplasma brabilience	

Classification based on computer simulated RFLP analysis of 16S rRNA genes (Wei *et al*., 2007:

After above classification systems numerous phytoplasmas were discovered worldwide which led to increase in number of 16Sr groups. However as collection of all phytoplasma strains as source of DNA hindered the attempts to classify and expand the 16Sr group classification. So the recent advances in bioinformatics were utilized by **Wei *et al*., 2007,** by the use of 800 phytoplasma sequences which were deposited to National Centre for Biotechnology Information (NCBI) nucleotide sequence database. So *in-silico* RFLP analysis led to the generation of new phytoplasma groups.

For expansion of 16Sr group level classification 16S rRNA gene sequences were taken online from NCBI nucleotide sequence database and kept in Microsoft Excel based mini-database. For cladistic analysis 16S rRNA sequences of 90 non-phytoplasma bacteria taxa and two cyanobacterial taxa were also reterived. Thereafter alignment of 16S rRNA sequences was done in CLUSTAL-X format

and each aligned sequence was trimmed to an approximately 1.25 Kb fragment (termed as F2nR2 region) that was bounded by two conserved nucleotide blocks corresponding to the annealing sites for phytoplasma universal primers and finally 524 sequences were selected. Aligned sequences were then digested *in-silico* with 17 restiction enzymes (used by **Lee *et al.*, 1998).** After *in-silico* restriction digestion, a virtual 3.0% agrose gel image plotted automatically. Then comparison of virtual RFLP pattern was done and similarity coefficient was calculated.

A total of 51 RFLP patterns were obtained and phylogenetic tree was constructed using 524 phytoplasma F2nR2 sequences and other non phytoplasma 16Sr RNA sequences and it was found that phytoplasma formed a monophyletic clade. A tree topography was then reconstructed from 524 phytoplasma sequence accessions. Three major branches and at least 14 subclades (indicated by Arabic numbers) were evident within phytoplasma clade.

Based on similarity coefficient threshold of greater than 0.9 with in group a total of 28 groups and greater than 100 subgroups were proposed as shown in **Table 8**.

Table 8: Classification of Phytoplasmas Based on *in silico* RFLP Analysis of 16S rRNA Gene Sequences

16 Sr. Groups	Strain	Reference
16 SrI: Aster yellows group		
I-A	**Aster yellow witches'-broom phytoplasma (AYWB) *rn*A**	***Bai et al.*, 2006**
I-A	**Aster yellow witches'-broom phytoplasma (AYWB) *rn*B**	**Bai *et al.*, 2006**
I-B	**Onion yellows phytoplasma mild strain (OY-M) rnA**	**Oshima *et al.*, 2004**
I-B	**Onion yellows phytoplasma mild strain (OY-M) rnB**	**Oshima *et al.*, 2004**
I-B	**'*Ca.* Phytoplasma asteria'**	**Lee *et al.*, 2004a**
I-C	**Clover phyllody phytoplasma strain CPh**	**2000, Gen Bank submission**
I-D	**Aster yellows phytoplasma strain PaWB**	**2003, Gen Bank submission**
I-E	**Blueberry stunt phytoplasma strain BBS3**	**2003, Gen Bank submission**
I-F	**Aster yellows phytoplasma strain ACLR-AY**	**2003, Gen Bank submission**
16SrII: Peanut WB group		
II-A	**Peanut witches' broom phytoplasma**	**Gundersen *et al.*, 1994**
II-B	**'*Ca.* Phytoplasma aurantifolia'**	**Zreik *et al.*, 1995**
II-C	**Cactus witches'-broom phytoplasma**	**Cai *et al.*, 2002**
II-D	**'*Ca.* Phytoplasma australasiae**	**White *et al.*, 1998**
16SrIII: X-disease group		
III-A	**Western X-disease phytoplasma**	**1999, GenBank submission**
III-B	**Clover yellow edge phytoplasma**	**1999, GenBank submission**
16SrIV: Coconut lethal yellows groups		
IV-A	**Coconut lethal yellowing phytoplasma (LYJ-C8)**	**Harrison *et al.*, 2002a**
IV-B	**Phytoplasam sp. Lfy5 (PE65)-Oaxaca**	**Harrison *et al.*, 2002b**
IV-D	**Carludovica palmate leaf yellowing phytoplasma**	**Cordova *et al.*, 2000**
16SrV: Elm yellows group		
V-A	**'*Ca.* Phytoplasma ulmi'**	**Lee *et al.*, 2004b**
V-B	**'*Ca* Pyhytoplasma ziziphi' strain JWB-G1**	**Jung *et al.*, 2003a**
V-C	**Alder yellows phytoplasma strain ALY 882**	**Lee *et al.*, 2004b**
V-G	**'*Ca.* Phytoplasma ziziphi'- related strain JWB-Korl**	**Jung *et al.*, 2003a**
16SrVI: Clover proliferation group		

VI-A	**'*Ca*. Phytoplasma trifolii'**	**Hiruki & WanG, 2004**
16SrVII: Ash yellows group		
	'*Ca*. Phytoplasma fraxini'	**Griffiths *et al*., 1999**
16SrVIII: Loofah witches' broom group		
VIII A	**Loofah witches' broom phytoplasma**	**2001, GenBank submission**
16SrIX: Pigeon pea witches' broom		
IX-A	**Pigeon pea witches'-broom phytoplasma**	**2000, GenBank submission**
IX-D	**'*Ca*. Phytoplasma phoenicium'**	**Verdin *et al*., 2003**
16SrX: Apple proliferation group		
X-A	**'*Ca*. Phytoplasma mail'**	**Seemuller & Schneider, 2004**
X-C	**'*Ca*. Phytoplasma pyri'**	**Seemuller & Schneider, 2004**
X-D	**'*Ca*. Phytoplasma spartii'**	**Marcone *et al*., 2004a**
X-F	**'*Ca*. Phytoplasma prunorum'**	**Seemuller & Schneider, 2004**
16SrXI: Rice yellow dwarf group		
XI-A	**'*Ca*. Phytoplasma oryzae'**	**Jung *et al*., 2003b**
16SrXII: Stolbur group		
XII-A	**'*Ca*. Phytoplasma solani'**	**Firrao *et al*., 2005 (incidental citation)**
XII-B	**'*Ca*. Phytoplasma australiense'**	**Davis *et al*., 1997**
XII-C	**Strawberry lethal yellows phytoplasma**	**Padovan *et al*., 2000b**
XII-D	**'*Ca*. Phytoplasma japonicum'**	**Sawayanagi *et al*., 1999**
XII-E	**'*Ca*. Phytoplasma fragariae'**	**Valiunas *et al*., 2006**
16SrXIII Mexican periwinkle virescence group		
XIII-A	**Mexican periwinkle virescence phytoplasma**	**2000, GenBank submission**
16SrXIV: Bermudagrass white leaf group		
XIV-A	**'*Ca*. Phytoplasma cynodonits'**	**Macrone *et al*., 2004b**
16SrXV: Hibiscus witches' broom group		
XV-A	**'*Ca*. Phytoplasma brasiliense'**	**Montano *et al*., 2001**
16SrXVI: Sugar cane yellow leaf syndrome group		
XVI-A	**'*Ca*. Phytoplasma graminis'**	**Arocha *et al*., 2005**
16SrXVII: Papaya bunchy top group		
XVII-A	**'*Ca*. Phytoplasma caricae'**	**Arocha *et al*., 2005**
16SrXVIII American (TX+NE) potato purple top wilt group		
XVIII-A	**'*Ca*. Phytoplasma americanum'**	**Lee *et al*., 2006**
16SrXVIII American (TX+NE) potato purple top wilt group		
XVIII-A	**'*Ca*. Phytoplasma castaneae'**	**Jung *et al*., 2002**
16SrXIX: Japanese chestnut witches'-broom group		
XIX-A	**'*Ca*. Phytoplasma rhamni'**	**Marcone *et al*., 2004a**
16SrXXI: Pine shoot proliferation group		
XXI-A	**'*Ca*. Phytoplasma pini'**	**Schneider *et al*., 2005**
16SrXXII: Nigerian coconut lethal decline (LDN) group		
XXII-A	**Phytoplasma sp. strain LDN**	**Tymon *et al*., 1998**
16SrXXIII: Buckland Valley grapevine yellows group		
XXIII-A	**Buckland valley grapevine yellow phytoplasma**	**Constable *et al*., 2002**
16SrXXIV: Sorghum bunchy shoot group		
XXIV-A	**Sorghum bunchy shoot phytoplasma**	**Blanche *et al*., 2003**
16SrXXV: Weeping tea tree witches'		
XXV-A	**Weeping tea witches'-broom phytoplasma**	**2002, GenBank submission**
16SrXXVI: Mauritius sugar cane yellows D3T1 group		
XXVII-A	**Sugar cane phytoplasma D3T1**	**2003, GenBank submission**
16SrXXVII: Mauritius sugar cane yellows D3T2 group		
XXVII-A	**Sugar cane phytoplasma D3T2**	**2003, GenBank submission)**
16SrXXVIII: Havana derbid phytoplasma group		
XXVIII-A	**Derbid phytoplasma**	**2004, GenBank submission**

Key Enzymes for Classification to Groups

Three key enzymes which could be utilized for classification of new phytoplasma strain to 16Sr groups were determined as *Mse*I, *Rsa*I and *Hinf*I.

A total of 28 groups and more than 100 subgroups were proposed. New groups contains 3 previously defined *Ca*. Phytoplasma species and 7 new potential species yet to be described. This provided a feasible means for extension of phytoplasma classification. The availability of a comprehensive set of phytoplasma 16SrRNA gene RFLP pattern types **(Lee *et al*., 1993, 1998, 2000)** has made possible the accurate and reliable identification, differentiation and classification of a broad array of phytoplasmas and has greatly stimulated and expanded phytoplasma research over the past decade. By utilizing *in-silco* RFLP analysis classification could be expanded from time to time.

CONCLUSIONS

Phytoplasmas are important plant pathogens causing economic losses to large number of agricultural and horticultural crops world-wide. Molecular based tools developed in past decade have permitted great advances in the diagnostics, identification and classification of phytoplasmas. Phytoplasma-specific generic primers developed on the basis of conserved 16S rRNA gene have universally been employed for amplification and subsequently RFLP analysis for their classification. International Committee on Systematic Bacteriology (ICSB) - Subcommittee on the Taxonomy of *Mollicutes* has agreed to adopt phylogeny based taxonomy for phytoplasmas due to the inability to culture them *in-vitro*. Comprehensive phylogenetic analysis has revealed that phytoplasma clade comprises greater than 20 subclades. There is consensus that each subclade or 16Sr group should represent atleast one a species. A provisional taxonomic system has been proposed to categorize phytoplasma as *Candidatus* Phytoplasma species. It is accepted by Phytoplasma/Spiroplasma Working Team **IRPCM, 2000,** that for a stable, reliable and valid classification each *Candidatus* Phytoplasma species should be described in terms of 16S rRNA gene sequence region, biological and phytopathological properties. Till now more than 26 *Candidatus* Phytoplasma species has been described and it is being found that a single 16Sr group may comprise more than one species.

- RFLP analysis of PCR-amplified 16S rRNA gene with restriction enzymes remains a valuable tool for studying phytoplasma diversity and classification.

- Till now the most accepted and stable classification is to describe phytoplasmas in '*Candidatus* phytoplasma species' rank which combines both molecular (16S rRNA gene sequence) as well as biological, phytopathological properties.

Future Prospects

- Extensive research is to be made to culture the phytoplasma *in-vitro* by trying

  various nutrition media so as to have binomial taxonomy for phytoplasma which relies on morphological and biochemical characteristics of organisms.
- More research data is to be generated for a stable taxonomy and classification

  system.
- Research related to pathogenicity of phytoplasmas needed to be done.

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