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Review Article

Phytoplasma classification: Taxonomy based on 16S ribosomal gene, is it enough?

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Abstract

The discovery in 1967, of a new group of plant pathogens related to bacteria led to the finding of pleomorphic, wall-less prokaryotes in the phloem of many plant species affected by yellows-type diseases. Following the application of molecular technologies the phylogeny of these prokaryotes was resolved and led to the new trivial name of "phytoplasma" and to the designation of a new taxon named '*Candidatus* phytoplasma'. The first comprehensive phytoplasma classification scheme was based on RFLP analysis of PCR-amplified 16S rDNA, providing a reliable means for the differentiation of a broad array of phytoplasmas and has become the most comprehensive and widely accepted phytoplasma classification system. This approach using RFLP analyses of PCR amplified 16S rDNA provides a simple, reliable and rapid mean for differentiation and identification of known phytoplasmas. A consensus for naming novel phytoplasmas was recommended by the IRPCM Phytoplasma/Spiroplasma Working Team-Phytoplasma Taxonomy Group that a '*Candidatus* Phytoplasma' species description should refer to a single, unique 16S rRNA gene sequence that has <97.5% similarity to that of any previously described '*Ca*. Phytoplasma' species. Because of the highly conserved nature of the 16S rDNA, many biologically or ecologically distinct phytoplasma strains, which may warrant designation of a new taxon may fail to meet the requirement. In this case, additional unique biological properties such as antibody specificity, host range and vector transmission specificity as well as other molecular criteria (gene) need to be included for speciation. However, because of the highly conserved nature of the 16S rDNA and of the not uncommon presence of 16S rDNA interoperon sequence heterogeneity, the classification based on '*Candidatus*' or on 16S ribosomal group does not always provide the molecular distinction necessary for phytoplasma strain characterization. Moreover, some additional tools for phylogenetic analyses and finer strain differentiation of phytoplasmas such as *rp, secY, tuf, groEL* genes, and the 16S-23S rRNA intergenic spacer region sequences have been used as supplementary tools selecting those providing the most useful and reliable taxonomic information in combination with 16SrDNA.

Keywords : Phytoplasma, classification, taxonomy, 16S ribosomal gene

Introduction

The discovery in 1967 (Doi *et al*.) of a new group of plant pathogens related to bacteria led to the finding of pleomorphic, wall-less prokaryotes in the phloem of many plant species affected by yellows-type diseases. The term mycoplasma-like organisms (MLOs) was first used to name these micro-organisms due to their morphological and ultrastructural similarity to mycoplasmas infecting animals (Fig. 1).

Following the application of molecular technologies the phylogeny of these prokaryotes was resolved and led to the designation of a new taxon named '*Candidatus* phytoplasma' (IRPCM, 2004) and to the new trivial name of "phytoplasma", the '*Candidatus*' term should be dropped only when biological characteristics could be used for their classification i.e. after consistent success in attempts to culture them in pure culture in cell free media (Bertaccini *et al*., 2010).

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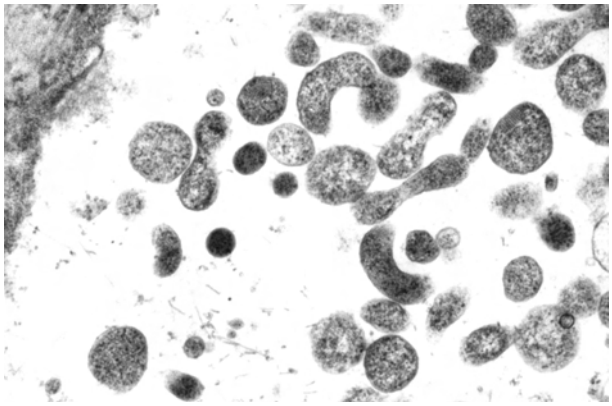

Figure 1. Electron micrograph of plemorphic phytoplasmas in a sieve tube (X 10,000)

Plants infected by phytoplasma exhibit an array of symptoms that suggests profound disturbances in the normal balance of growth regulators (Fig. 2). They are transmitted by insects belonging to the families Cicadellidae, Cixidae, Psyllidae, Delphacidae and Derbidae (Weintraub and Beanland, 2006).

Recent molecular data on phytoplasmas have provided considerable insights into their diversity and genetic interrelationships that are the basis for several comprehensive studies on phytoplasma phylogeny and taxonomy (Hogenhout *et al*., 2008). Sequence analysis of 16S rDNA have shown that phytoplasmas constitute a coherent taxon; in the phytoplasma clade groups and subgroups were delineated that are generally consistent with '*Candidatus*' species (Tables 1 and 2).

The first comprehensive phytoplasma classification scheme was based on restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR)-amplified 16S rDNA (Lee *et al*., 1998), providing a reliable means for the differentiation of a broad array of phytoplasmas and has become the most comprehensive and widely accepted phytoplasma classification system (reviewed in Bertaccini and Duduk, 2009).

Sensitive and accurate detection of these micro-organisms is a prerequisite for the management of phytoplasma-associated diseases. The nucleic acid techniques based on polymerase chain reaction (PCR) procedures developed in the last 20 years are now used routinely and are adequate for detecting phytoplasmas. In particular the techniques enhanced detection of phytoplasma presence in plant propagation material and allow identification of putative insect vectors, thus helping in preventing the spread of the diseases and their economic impact.

Figure 2. Symptoms associated with phytoplasma presence in (top left to right): Jujube in China, purple coneflower in Italy, and (bottom left to right): rubus in Germany, tomato in Italy and poinsettia (plant on the right).

Table 1. '*Candidatus* Phytoplasma' nomenclature validly published compared with 16S rRNA classification based on Lee *et al.* (1998) when possible.

'*Candidatus* Phytoplasma' (disease, acronym)	16Sr subgroup[1]	GenBank Acc. no.	Reference
'*Candidatus* Phytoplasma asteris' (aster yellows, AY)	**16SrI-B**	M30790	Lee *et al*. (2004a)
'*Ca.* P. aurantifolia' (witches' broom of lime, WBDL)	**16SrII-B**	U15442	Zreik *et al*. (1995)
'*Ca.* P. ulmi' (elm yellows, EY)	**16SrV-A**	AY197655	Lee *et al*. (2004b)
'*Ca.* P. ziziphi' (Jujube witches' broom, JWB-G1)	**16SrV-B**	AB052876	Jung *et al*. (2003a)
'*Ca*. P. rubi' (Rubus stunt, RuS)	**16SrV-E**	AY197648	Malembic-Maher *et al.* (2010)
'*Ca.* P. trifolii' (clover proliferation, CP)	**16SrVI-A**	AY390261	Hiruki and Wang (2004)
'*Ca.* P. fraxini' (ash yellows, AshY)	**16SrVII-A**	AF092209	Griffiths *et al*. (1999)
'*Ca.* P. phoenicium' (almond witches' broom, ALWB)	**16SrIX-B**	AF515636 AF515637	Verdin *et al*. (2002)
'*Ca.* P. mali' (apple proliferation, AP)	**16SrX-A**	AJ542541	Seemüller and Schneider (2004)
'*Ca.* P. prunorum' (European stone fruit yellows, ESFY)	**16SrX-B**	AJ542544	Seemüller and Schneider (2004)
'*Ca.* P. pyri' (pear decline, PD)	**16SrX-C**	AJ542543	Seemüller and Schneider (2004)
'*Ca.* P. spartii' (spartium witches' broom, SpaWB)	**16SrX-D**	X92869	Marcone *et al*. (2004a)
'*Ca.* P. oryzae' (rice yellow dwarf, RYD)	**16SrXI-A**	AB052873	Jung *et al*. (2003b)
'*Ca.* P. australiense' (Australian grapevine yellows, AUSGY)	**16SrXII-B**	L76865	Davis *et al*. (1997)
'*Ca.* P. cynodontis' (bermudagrass white leaf, BGWL)	**16SrXIV-A**	AJ550984	Marcone *et al*. (2004b)
'*Ca*. P. brasiliense' (hibiscus witches' broom, HiWB)	**16SrXV-A**	AF147708	Montano *et al.* (2001)
'*Ca*. P. graminis' (sugarcane yellow leaf, SYL)	**16SrXVI-A**	AY725228	Arocha *et al.* (2005)
'*Ca*. P. caricae' (papaya bunchy top, PBT)	**16SrXVII-A**	AY725234	Arocha *et al.* (2005)
'*Ca*. P. americanum' (American potato purple top wilt, APPTW)	**16SrXVIII-A**	DQ174122	Lee *et al.* (2006a)
'*Ca*. P. omanense' (cassia witches' broom, CaWB)	**16SXIX-A**	EF666051	Al-Saady *et al.* (2008)
'*Ca.* P. japonicum' hydrangea phyllody		AB010425	Sawayanagi *et al*. (1999)
'*Ca.* P. castaneae' chestnut witches' broom	***16SrXIX***	AB054986	Jung *et al.* (2002)
'*Ca*. P. rhamni' (Rhamnus witches' broom, RaWB)	***16SrXX***	AJ583009	Marcone *et al*. (2004a)
'*Ca*. P. pini' (*Pinus sylvestris* yellows, PinY)	***16SrXXI***	AJ310849	Schneider *et al*. (2005)
'*Ca*. P. allocasuarinae' (allocasuarina yellows, AllocY)		AY135523 AY135524	Marcone *et al*. (2004a)
'*Ca*. P. fragariae' (strawberry yellows, StrawY)		DQ086423	Valiunas *et al*. (2006)
'*Ca*. P. lycopersici' ('Brote grande' tomato, TBG)		EF199549	Arocha *et al.* (2007)
'*Ca*. P. tamaricis' (salt cedar witches' broom, SaltCWB)		FJ432664	Zhao *et al.* (2009)
'*Ca*. P. costaricanum' (soybean decline, SoyD)		HQ225630	Lee *et al*. (2011)

[1], in Italics groups designated by Wei *et al*. (2007).

Phytoplasma identification and classification

In the 1990's, following the first cloning of phytoplasma DNA (Kirkpatrick *et al*., 1987), nucleic acid-based probes were applied to detect and differentiate phytoplasmas (Lee and Davis, 1988; Bertaccini *et al*., 1990a; Bonnet *et al*., 1990; Harrison *et al*., 1992, Prince *et al*., 1993; Davis *et al*., 2003) and provided the first evidences of genetic differences in the phytoplasma DNA among strains derived from different hosts or from different geographical locations (Lee *et al*., 1992; Bertaccini *et al*., 1990b;

Table 2. 16S rRNA subgroup distinguished based on Lee *et al.* (1998)

16Sr subgroup	Phytoplasma strain	GenBank Acc. no.	Reference
16SrI: Aster yellows			
I-A	Aster yellows witches' broom (AYWB)	NC_007716	Bai *et al.* (2006)
I-A	Tomato big bud (BB)	L33760	Lee *et al.* (1992)
I-B	Onion yellows mild strain (OY-M)	NC_005303	Oshima *et al.* (2004)
I-C	Clover phyllody (CPh)	AF222065	Lee *et al.* (2004a)
I-D	Paulownia witches' broom (PaWB)	AY265206	Lee *et al.* (2004a)
I-E	Blueberry stunt (BBS3)	AY265213	Lee *et al.* (2004a)
I-F	Aster yellows from apricot (A-AY)	AY265211	Lee *et al.* (2004a)
I-I	Strawberry witches' broom (Strawb1)	U96614	Jomantiene *et al.* (1998a,b)
I-K	Strawberry witches' broom (strawb2)	U96616	Jomantiene *et al.* (1998a,b)
I-L	Aster yellows (AV2192)	AY180957	Lee *et al.* (2003)
I-M	Aster yellows (AVUT)	AY265209	Lee *et al.* (2004a)
I-N	Aster yellows (IoWB)	AY265205	Lee *et al.* (2004a)
I-O	Soybean purple stem (SPS)	AF268405	Lee *et al.* (2002)
I-P	Aster yellows from *Populus*	AF503568	Šeruga *et al.* (2003)
I-Q	Cherry little leaf (ChLL)	–	Valiunas *et al.* (2005)
I-R	Strawberry phylloid fruit (StrawbPhF)	AY102275	Jomantiene *et al.* (2002a)
16SrII: Peanut WB			
II-A	Peanut witches' broom (PnWB)	L33765	Gundersen *et al.* (1994)
II-C	Faba bean phyllody (FBP)	X83432	Schneider *et al.* (1995)
II-D	Sweet potato little leaf (SPLL)	AJ289193	Gibb *et al.* (1995)
II-E	*Pichris echioides* phyllody (PEY)	Y16393	Seemûller *et al.* (1998a)
II-F	Cotton phyllody (CoP)	EF186827	Khan *et al.* (2002)
16SrIII: X-disease			
III-A, '*Ca*. P. pruni'★	Western X-disease (WX)	AF533231	Liefting and Kirkpatrick (2003)
III-B	Clover yellow edge (CYE)	L33766 8	Gundersen *et al.* (1994)
III-C	Pecan bunch (PB)	EF186807	Martini *et al.* (2007)
III-D	Goldenrod yellows (GR1)	EF186810	Martini *et al.* (2007)
III-E	Spiraea stunt (SP1)	AF190228	Lee *et al.* (1998)
III-F	Milkweed yellows (MW1)	AF510724	Lee *et al.* (1998)
III-G	Walnut witches' broom (WWB)	AF190226	Lee *et al.* (1998)
		AF190227	
III-H	Poinsettia branch-inducing (PoiBI)	AF190223	Lee *et al.* (1997a)
III-I	Virginia grapevine yellows (VGYIII)	AF060875	Davis *et al.* (1998)
III-J	Chayote witches' broom (ChWBIII)	AF147706 AF1477067	Montano *et al.* (2000)
III-K	Strawberry leafy fruit	AF274876	Jomantiene and Davis (2000) (GenBank submission)

Table 2. 16S rRNA subgroup distinguished based on Lee *et al.* (1998)

16Sr subgroup	Phytoplasma strain	GenBank Acc. no.	Reference
III-L	Cassava frog skin disease	EU346761	Alvarez *et al.* (2009)
III-P	Dandelion virescence	AF370119 AF370120	Jomantiene and Davis (2001) (GenBank submission)
III-Q	Black raspberry witches' broom (BRWB7)	AF302841	Davis *et al.* (2001)
III-T	Sweet and sour cherry (ChD)	FJ231728	Valiunas *et al.* (2009)
III-U	Cirsium white leaf (CWL)	AF373106 AF373105	Mello *et al.* (2011)
16SrIV: Coconut lethal yellows			
IV-A, '*Ca*. P. palmae'★	Coconut lethal yellowing (LYJ-C8)	AF498307	Harrison *et al.* (2002)
IV-B	Yucatan coconut lethal decline (LDY)	U18753	Harrison *et al.* (1994)
IV-C, '*Ca*. P. cocostanzianae'★ '*Ca*. P. cocosnigeriae'★	Tanzanian coconut lethal decline (LDT)	X80117	Harrison *et al.* (1994)
16SrV: Elm yellows			
V-C	Alder yellows (ALY882)	AY197642	Lee *et al.* (2004b)
V-C, '*Ca*. P. vitis'★	Flavescence dorée (FD-C)	X76560	Daire *et al.* (1992)
V-D, '*Ca*. P. vitis'★	Flavescence dorée (FD-D)	AJ548787	Martini *et al.* (1999); Torres *et al.* (2005)
16SrVI: Clover proliferation			
VI-B	Fragaria multicipita	AF036354	Jomantiene *et al.* (1998a)
VI-C	Illinois Elm Yellows (ILEY)	AF268895 AF409069 AF409070	Jacobs *et al.* (2003)
16SrVII: Ash yellows			
VII-B	Erigeron witches' broom	AY034608	Barros *et al.* (2002)
16SrVIII: Loofah witches' broom			
VIII-A, '*Ca*. P. luffae'★	Loofah witches' broom	AF086621	Ho *et al.* (2001)
16SrIX: Pigeon pea witches' broom			
IX-A	Pigeon pea witches' broom	AF248957	Lee *et al.* (1998)
IX-C	Naxos periwinkle virescence	–	Heinrich *et al.* (2001)
16SrXI: Rice yellow dwarf			
XI-B	Sugarcane white leaf SCWL	X76432	Lee *et al.* (1997b)
XI-C	Leafhopper-borne BVK	X76429	Seemüller *et al.* (1994)
16SrXII: Stolbur			
XII-A, '*Ca*. P. solani'★	Stolbur STOL (Capsicum annum)	X76427	Seemüller *et al.* (1994)
16SrXIII: Mexican periwinkle virescence			
XIII-A	Mexican periwinkle virescence (MPV)	AF248960	Lee *et al.* (1998)
XIII-B	Strawberry green petal	U96616	Jomantiene *et al.* (1998b)

★'Candidatus' names proposed at the X International Congress of the International Organization of Mycoplasmology, 1994, held in Bordeaux, France, but not yet formally described as requested by the taxonomy committee, are reported here as incidental citations which do not constitute prior citations, according to rule 28b of the bacteriological code (Lapage *et al.*, 1992).

1993). Moreover genomic sequence-specific oligonucleotides developed for diagnostic purposes using generic or broad-spectrum primers designed based on 16S rDNA (Ahrens *et al*., 1993; Lee *et al*., 1993; Namba *et al*., 1993) allow detection of a wide array of phytoplasmas associated with plants and insects. Several universal and many phytoplasma group specific primers have been designed for routine detection of phytoplasmas on 16S ribosomal RNA gene. The main goal of each protocol was to concentrate phytoplasma DNA while reducing enzyme-inhibitory plant polyphenolic and polysaccharide molecules. This is generally attained by including a phytoplasma enrichment step. However nested-PCR assay, designed to increase both sensitivity and specificity, is indispensable for the amplification of phytoplasmas from samples in which unusually low titers, or inhibitors are present that may interfere the PCR efficacy (Bertaccini *et al*., 1992a; Gundersen *et al*., 1994; Heinrich *et al*., 2001). Nested-PCR is performed by preliminary amplification using a universal primers pair followed by second amplification using a second universal primer pair. By using a universal primer pair followed by PCR using a group-specific primer pair, nested-PCR is capable of detection of dual or multiple phytoplasmas present in the infected tissues in case of mixed infection (Lee *et al*., 1994). Differentiation of putative phytoplasmas now is routinely carried out on 16S rRNA gene that must be accomplished through Restriction Fragment Length Polymorphism (RFLP) analysis of PCR amplified DNA sequences using a number of endonuclease restriction enzymes (Lee *et al*., 1998). Because the RFLP patterns characteristics of each phytoplasmas are conserved, unknown phytoplasmas can be identified by comparing the patterns of the unknown with the available RFLP patterns for known phytoplasmas without co-analyses of all reference representative phytoplasmas (Lee *et al*., 1998; Wei *et al*., 2007, 2008).

RFLP analyses of 16S rDNA nested PCR products from 34 representative phytoplasma strains with 17 restriction enzymes was used by Lee *et al*. in 1998 to differentiate various phytoplasmas by their distinct RFLP patterns. Based on similarity coefficients derived from RFLP analyses, the 34 phytoplasma strains were differentiated into 14 major groups (termed 16Sr groups) and 32 sub-groups. By including additional groups and sub-groups from which RFLP analyses of available 16S rDNA sequence data groups and sub-groups were proposed for classification (Tables 1 and 2). The phytoplasma 16Sr groups has been shown to be consistent with the phylogenetic groups (clades) defined by phylogenetic analysis of near-full-length 16S rRNA gene sequences, indicating that the RFLP-based groups are phylogenetically valid. The approach using RFLP analyses of PCR amplified 16S rDNA provides a simple, reliable and rapid means for differentiation and identification of known phytoplasmas.

For finer differentiation of phytoplasmas, additional genetic markers such as ribosomal protein (rp) genes, secY, tuf, groEL and the 16S-23S rRNA intergenic spacer region sequences have been used as supplementary tools (Lee *et al*., 1994, 2004a,b, 2006b, 2010; Martini *et al*., 2002, 2007; Schneider *et al*., 1997; Mitrović *et al*., 2011; Smart *et al*., 1996). Finer subgroup delineation could be achieved by combining RFLP analyses of 16S rRNA and rp gene sequences: the subgroups recognized by these methods were consistent with the subclusters identified by analysis of phytoplasma genomes through dot and Southern hybridizations using a number of cloned phytoplasma DNA probes (Lee *et al*., 1992, 1998; Gundersen *et al*., 1996; Martini *et al*., 2007). A consensus for naming novel phytoplasmas was reached and recommended by the IRPCM Phytoplasma/Spiroplasma Working Team-Phytoplasma Taxonomy Group (IRPCM, 2004) that "a '*Candidatus* Phytoplasma' species description should refer to a single, unique 16S rRNA gene sequence (>1200 bp)", and "a strain can be recognized as a novel '*Ca*. Phytoplasma' species if its 16S rRNA gene sequence has <97.5% similarity to that of any previously described '*Ca*. Phytoplasma' species". Because of the highly conserved nature of the 16S rRNA gene, many biologically or ecologically distinct phytoplasma strains, which may warrant designation of a new taxon but may fail to meet the requirement of sharing <97.5% sequence similarity with existing '*Ca*. Phytoplasma', cannot be readily differentiated and classified. In this case, additional unique biological properties such as antibody specificity, host range and vector transmission specificity as well as other molecular criteria (gene) need to be included for speciation (Seemüller and Schneider, 2004). '*Candidatus* Phytoplasma' validly published are now 29 (Table 1). In these year a comprehensive collection of micropropagated phytoplasmas strains was established and made available to the scientific community for research purposes, the collection

comprises strains maintained in periwinkle, but also in other host plants (Bertaccini *et al*., 1992; Bertaccini, 2007, 2010) and is the reference strains collection for phytoplasmas described either at the '*Candidatus*' level or at the 16S RDNA group/subgroup level.

However the classification based on '*Candidatus*' or on 16S ribosomal group does not always provide the molecular distinction necessary for phytoplasma strain characterization for epidemiological studies towards disease control. At least the subgroup designation is necessary (Table 2) in some cases in fact quarantine pathogens such those associated with 'flavescence dorée' and stolbur the subgroup assignment is necessary although not supported yet by approved taxonomy.

Moreover because of the highly conserved nature of the 16S rRNA gene and of the not uncommon presence of 16S rDNA interoperon sequence heterogeneity (Schneider and Seemüller, 1994; Liefting *et al*., 1996; Lee *et al*., 1998; Jomantiene *et al*., 2002b; Davis *et al*., 2003; Jung *et al*., 2003c; Duduk *et al*., 2009), some additional tools for phylogenetic analyses and finer strain differentiation of this heterogeneous group are needed and multilocus sequence determination, using other genes already employed for phytoplasma characterization (Schaff *et al*., 1992; Lorenz *et al*., 1995; Jarausch *et al*., 2000; Marcone *et al*., 2000; Botti and Bertaccini, 2003, 2007; Langer and Maixner, 2004; Arnaud *et al*., 2007; Danet *et al*., 2011) selecting those providing the most useful and reliable taxonomic information in combination with 16S ribosomal gene. Phytoplasma classification following rules defined and shared among scientists to produce a reliable worldwide recognized classification system that allow not only taxonomic differentiation but also epidemic studies will be among the best tool towards phytoplasma associated diseases control.

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