ISSN: 2634 - 8845

Journal of Earth and Environmental Science Research

SCIENTIFIC Research and Community

Review Article

Open Access

Genomics of 'ca' Phytoplasma Vitis 'FD' and Related Strains in Silico

Gabor Zs Gyulai[1], Renee P Malone[2], Antal Szoke[1] and Gabor Gyulai[1*]

[1]MATE University, Institute of GBI, 2100 Gödöllő, Hungary

[2]College of Science and Health, Technological University, Grangegorman, Dublin 7, Ireland

ABSTRACT

The current achievements and limitations of the genetics and genomics of 'ca. Phytoplasma vitis Flavescence Dorée phytoplasma 'FDp' are reviewed and discussed here. The name originated from the Fr. flavescence (yellowing), and Fr. dorée (golden), the 'ca.' prefix stands for candidatus. The genetics of related Phytoplasma and Mycoplasma isolates are also discussed. Complete Phytoplasma genomes were compared in silico to follow the evolutionary scaled gene-loss and genome reductions. DNA sequences of Prophages and PMUs (Potential Mobile Units) of Phytoplasma genomes were analyzed. The Phytoplasma GeneBank entries and the possible molecular cross reactions are discussed. Sequence parts of Phytoplasma strains were found to obviously show sequence similarities to bacteria and plant organelle genomes of cpDNA and mtDNA which have prokaryotic origins from α-ProteoBacteria. The continuous horizontal gene transfers (HGT; i.e., influx of organellar DNA to nuclear genome; cytonuclear interactions) from plant organelles cpDNA and mtDNA to nuclear genomes (2n), including genes of 16S rRNA, ftsH and all homolog (ortholog and paralog) and analog genes were suggested to provide multiplied sequence targets with putative cross reactions to Phytoplasma identification. TimeTree of Life analysis of two infected host plants of Vitis and Solanum attractive to 'ca. Ph. vitis FD' and 'ca. Ph. solani BN' showed >60 MY (Vitis) and >150 MY (Solanum) times in the history of evolution which indicated different time period exposed to Phytoplasma infections. The data presented here highlights the importance of the identification of further Phytoplasma secreted proteins, the need for new serological and plant symptoms analyses, the use of Microgenomics by single cell Laser MicroDissection (LMD) technology, single cell genomics (CSG), and the possibility of in vitro cultivation of 'ca.' Phytoplasma strains to elimination 'ca.' prefix.

***Corresponding author**

Gabor Gyulai, MATE University, Institute of GBI, 2100 Gödöllő, Hungary.

Received: February 12, 2026; **Accepted:** February 16, 2026; **Published:** February 28, 2026

Mycoplasma and Phytoplasma Strains

Mycoplasma and Phytoplasma strains and isolates of Phylum Firmicutes of low-G+C, Gram-positive Eubacteria; Class Mollicutes, Genus Mycoplasma, >250 taxa (https://lpsn.dsmz.de) are Gram-positive, aerobic, single celled bacteria, which are the smallest host-free living organism (0.1–0.8 µm in size) carrying the smallest genomes of bacteria (0.5–1.3 x 106 bpDNA) comprising of central DNA ("chromosomes") and >5-10 plasmids (Table 1). The unique cell structure lacks the rigid bacterial cell wall [1,2]. Most Mycoplasma strains are parasite and invasive endosymbiont causing Human diseases [3-5].

The small genomes of Mycoplasmas and Phytoplasmas are the result of adaptation to the infected host organisms which provide metabolites to the endosymbiont bacteria leading to a continuous gene-loss and genome reduction by elimination of function-lost genes [6-9]. Gene-loss comprises gene clusters of the tricarboxylic acid cycle (TCA), sterol biosynthesis, fatty acid biosynthesis, de novo nucleotide synthesis, and genes of biosynthesis of most amino acids [2,8].

The free-living bacteria with a rigid cell wall of either Gram(+) or Gram(-) have larger genomes. E.g., the first sequenced genome of Haemophilus influenzae [Syn.: Bacillus influenzae] with 1 890 662 bpDNA genome (GC% 38; CP085952.1); Agrobacterium tumefaciens (2 074 782 bpDNA genome; GC% 58.5; NC_003063); Mycobacterium tuberculosis (4 411 532 bpDNA genome; GC% 65.5; AL123456.3); and Escherichia coli strain K-12 (4 639 221 bpDNA genome; GC% 51; U00096.3) [10,11].

Phytoplasma Strains

Phytoplasmas (i.e., 'plant Mycoplasmas') are insect-transmitted, also cell wall-less bacteria (https://lpsn.dsmz.de) which are symbiont of plants and insects [2,12]. By systematic, Phytoplasmas were grouped to Mycoplasma in 1929, and the term Phytoplasma was recommended to use from 1992, suggested by the International Committee on Systematic Bacteriology [13,14]. The 'ca.' prefix (Table 1) is used from 1994 and stands for all organisms which cannot be isolated and cultured in aseptic laboratory conditions in vitro from outside their host plants [15,16].

Table 1. Comparison of genome sizes of some Mycoplasma and ca. Phytoplasma (ca. Ph.) strains in order of genome sizes (>27 complete genome) available at GeneBank (NCBI/genome; KEGG, https://www.genome.jp/kegg/genome/) with indications of GC% content and the submitted years. Symbol ♦ indicates the first complete Phytoplasma genome sequenced [7]. DDicots and MMonocots of infected Angiosperm plants are indicated. Related Phytoplasmas are labeled*,◊ [17,18].

Mycoplasma strains

- 580 071 bpDNA Mycoplasma genitalium [CP159789.1; GC% 31.5]
- 817 125 bpDNA Mycoplasm(oides) pneumoniae

[LR214945.1; GC% 40]

Phytoplasma strains

Gymnosperms

- 474 100 bpDNA ca. Ph. pini [GCA_007821455.1; GC% ?; 2019]

Angiosperms

- 498 922 bpDNA, ca. Ph. cynodontis (Bermudagrass, C. dactylon) [CP126225.1; GC% 21; 2023]^{M}
- 654 223 bpDNA, ca. Ph. vitis* (GYP) [CP097583; GC% 21.5; 2022]^{D}
- 704 525 bpDNA, ca. Ph. solani* (CPS, CaPsol) [CP155828.1; GC% 26; 2024]^{D}
- 751 320 bpDNA, ca. Ph. solani and Vitis 'Bois Noir'* (BN) [CP103788.1]^{D}
- 762 251 bpDNA, ca. Ph. rubi (Rubus stunt disease) [GCA_026821955.1; GC% 23; 2023]^{D}
- 769 143 bpDNA, ca. Ph. luffae [CP054393.1; GC% 23.5; 2021]^{D}
- 772 691 bpDNA, ca. Ph. Aster◊ yellows group (AYP) [e.g., CP128414.1; GC% 28; 2024]^{D}
- 853 092 bpDNA, ca. Ph. onion◊ yellows mild (OYM of AYP) [AP006628; GC% 28; 2004]$^{M(\blacklozenge)}$
- 959 779 bpDNA, ca. Ph. australiense (strawberry) [CP002548.1; GC% 27; 2013]^{D}
- 879 324 bpDNA, ca. Ph. australiense; Vitis (AusGY) and papaya [AM422018; GC% 27; 2008]^{D}
- 891 641 bpDNA, ca. Ph Paulownia◊ witches'-broom (PWB of AYP) str. 'Zhengzhou' [CP066882; GC% 27.5; 2021]^{D}
- 959 779 bpDNA, ca. Ph. strawberry lethal yellows (CPA) [CP002548; GC% 27; 3013]^{D}

The first documented Phytoplasma infection was named MLO (Mycoplasma-Like Organism) by Doi et al. who studied four plant species infected by plant yellow diseases by TEM (transmission electron microscopy >20.000 x magnification) of MDD, Mulberry dwarf disease (Morus alba used for feeding silkworm); CaPsol: ca. Phytoplasma solani (Syn.: Potato [Solanum] witches'-broom disease); AYP: Aster Yellows Phytoplasma (infected mainly Asteraceae and Umbelliferae species); and PaWB Paulownia witches'-broom disease (Lamiales) [19].

A thousand year old paint of peony (Peonia ssp) infected by phytoplasma is documented and exhibited in Museum of the Imperial Collections, Sannomaru-Shozokan, China [20].

Mulberry dwarf disease (MDD) was observed first in Japan during the Tokugawa Period (1603–1868) [2].

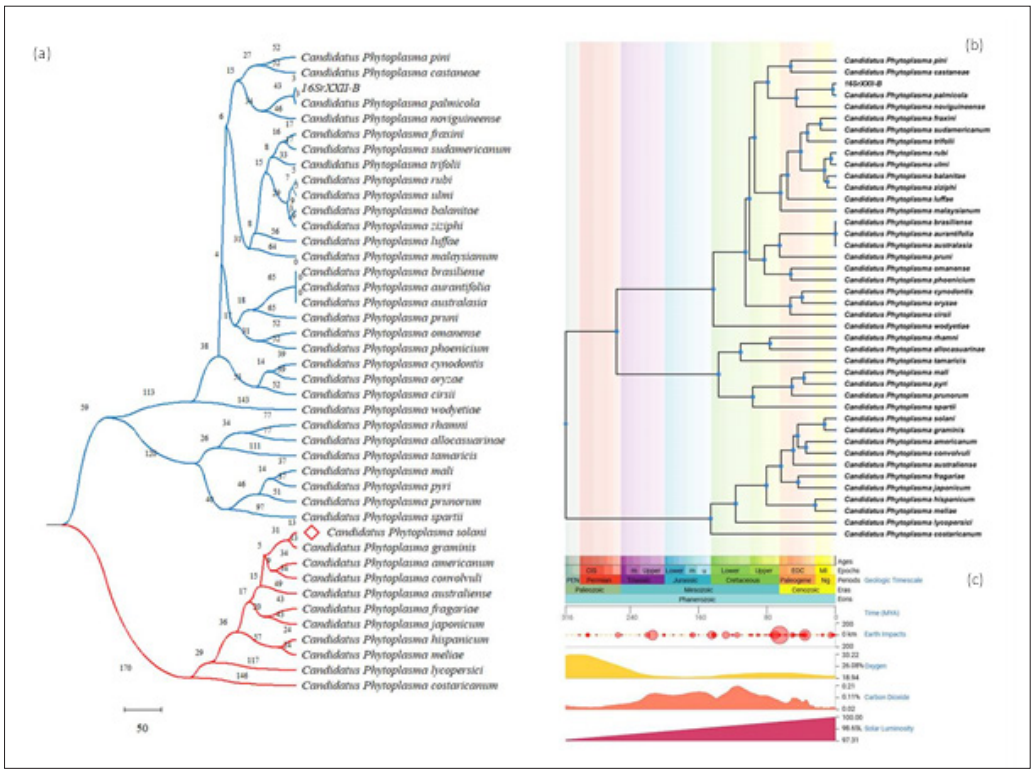

Figure 1: (a) Phylogenetic tree (NJ) of reedited tree (b) by MEGA7 of ca. Phytoplasma entries (42 of GeneBank, NCBI) [21]. The ca. Ph. CaPsol (◊), branch lengths, and genetic distance (scale 50) are indicated. (c) TimeTree of Life5 (TToL5 timetree.org) cladogram with Geological Timescale and changes in Divergence times (MYA), and changes in the environmental indicators O2, CO_2 and solar energy [52].

Plant dwarfism was reconstructed to appear in Japan about 1870 [22]. A reconstructed observation of Phytoplasma bronze leaf wilt of coconut was found in Africa (Nigeria) in about 1917 [23]. Numerous Monocots and Dicots of Angiosperm plants infected by Phytoplasmas have been registered (Table 1), however Gymnosperm trees (e.g., Pinus) [24] have reported rarely (Table 1) due to a possible size blocking of the narrow pore sizes sieve tubes (50-85 nm) of Gymnosperm homoxyl wood phloem tissues compared to Phytoplasma cell sizes (200-800 nm) [the pore sizes of seeve tubes of Dicots is 1-14 µm] [25]. Phytoplasma infection has reached ferns (Pteridophyta) [20,26].

Symptoms of Phytoplasma Vitis Infections

The visual symptoms; transmission electron microscopy (TEM) and molecular technologies of ELISA (https://loewe-info.com; https://ephyrabiosciences.com; https://www.agdia-emea.com); RFLP;

PCR (https://algimed-techno.com/en) and RT-PCR, Long-PCR, nested-PCR, mPCR, dPCR; targeted locus amplification (TLA); phytoplasma-responsive sRNAs (miRNAs and siRNAs) isolations; CRISP RNA technologies; microflow-chip (microfluidic) PCR; and microarrays-GeneChip technologies (Affymetrix, Inc.) have been used [2,8,19,20,27-35].

The symptoms on Phytoplasma infected plants (over 1000 species; in Hungary >30 species have been reported; show virus infection-like diseases [36-38]. Symptomps of plant Phytoplasma infections (phytoplasmosis) interfere with plant body development, such as the plant dwarfism; formation of bunchy fibrous secondary roots; brooming (clustering of branches), i.e., witches'-broom (e.g., on Poinsettia) with excessive tillering; bolting (growth of elongated stalks) with stem internode shortening; purple top of the stems; rolling and little leaves; leaf chlorosis (yellowing of leaves and stems due to chlorophyll degradation); flower virescence (greening of non-green flower's petals) [1,20,33]; phyllody (leaf-like flower petals and sepals as in Hydrangea phyllody); fruit abnormalities (e.g., Rubus) and necrosis of phloem sieve tubes and tissues, have been reported [20,22,39-41].

Phytoplasma Spread Insect Vectors

Background. The first invasive grape aphid (Phylloxera vitifoliae) feeding on Vitis roots and leaf galls appeared in Europe in 1850 introduced from Americas. It was followed by a devastating epidemic to viticulture and viniculture between 1863-1885 [42] of grape growing countries from Portugal to Hungary (in there with a later epidemic 1875-1914).

FD of FDP (Flavescence Dorée Phytoplasmas) (Table 1) transmitted by Scaphoideus titanus was accidentally introduced from North America to Europe observed first in France 1957 [42] or 1958 (https://www.forestpests.eu/pest/scaphoideus-titanus), later in Hungary 2007 [43-45]; Ukraine 2018; Spain (Madeira Island) 2020; and Germany (Baden-Württemberg) 2024 [46]. [To escape confusion, phytophtora is an invasive fungus Phytophthora infestans, e.g. on potato (potato late blight) with epidemy in Europe (Ireland), 1845-1852].

The phloem tissue sap-feeding insects (Hemiptera) like Sc. titanus transmit the saliva of salivary gland with the endosymbiont Phytoplasma bacteria during feeding, and disperse it from plant to plant [47]. The vector insects are numerous: aphids (of >5 000 species); whiteflies (of >1 500 species); thrips (of >7 700 species) e.g., Aphis nerii; psyllids (Syn.: jumping plant lice) of >50 pest species (and >4 species used in biocontrol) (www.psyllids.org), e.g., Psylla alni; leafhoppers (of >20 000 species), e.g., Scaphoideus titanus of Cicadellidae); planthoppers (of >12 500 species); cicadas (of >3 000 species); and flies (of >150 000 species), e.g., tsetse fly Glossina of 34 species [46,48].

GYP, Grape Yellows Phytoplasma strains are transmitted mainly by Fulgoromorpha spp., Hyalesthes obsoletus*, Reptalus panzeri, and Euscelis** incises [41].

AYP, Aster Yellows Phytoplasmas (Table 1) infecting >300 plant species of 38 families (mainly of Asteraceae and Umbelliferae) are transmitted mainly by Aster leafhopper (Macrosteles quadrilineatus) to mainly sunflowers, onion, lettuce, tomato, celery and maize (i.e., AY-WB and Maize Bushy Stunt Phytoplasma (MBSP) [2].
CaPsol, ca. Phytoplasma solani (Table 1) (Syn.: Potato witches'-broom disease) is transmitted mainly by Hyalesthes obsoletus*, Dictyophara europaea, and Euscelis** variegates of the total 35 insect vectors.

PaWB, Paulownia Witches'-Broom Phytoplasma spreads mainly by the stink bug Halyomorpha halys [49].

Phytoplasma bacteria also spread by vegetative propagation (e.g., budding or grafting) due to the non-sterilized tools.

Phytoplasma Vitis Strains Genetics Concerns

Phytoplasma strains have unique life cycles by replicating of genome in both the infected plants and in the vector insects [2,12].

Similar to Mycoplasmas the Phytoplasma genomes have low GC% levels, and plasmids have lower levels. E.g., GC% level of genomic DNA of ca. Ph. asteris (AYP) (CP128414.1) (Table 1) is 28% compared to the plasmid's (5 617 bpDNA encoding eight genes) with 22.5 GC% (CP128415.1) (direct submission NCBI, 2020) [9].

The 'ca.' Phytoplasma vitis 'FD' of FDP (Flavescence Dorée Phytoplasmas) strain CH (CP097583.1) (16SrV, Elm yellows group) of GYP (Grape Yellows Phytoplasma complex) including ca. Phytoplasma vitis (Table 1) were registered by Marzorati et al. [14,27,50]. It threats the global viticulture and viniculture (https://portal.nebih.gov.hu; https://www.genome.jp/kegg/genome/;) [51].

Molecular identifications of strains are mainly based on the gene sequences of structural 16S rRNAs of ribosomes (Figure 2), or 16S rRNA genes + methionine aminopeptidase (map) gene sequences [42].

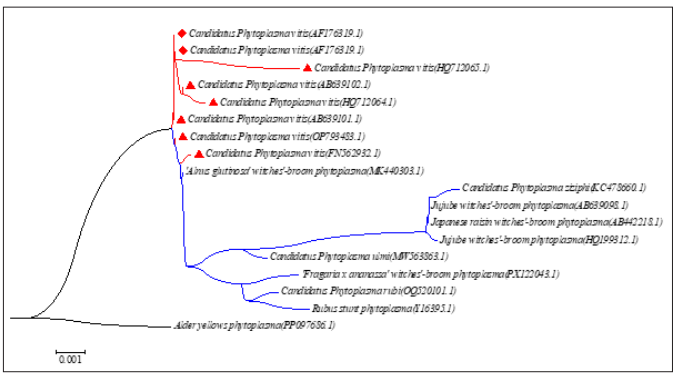

Figure 2: Phylogenetic NJ tree of DNA sequences of 16S rRNA genes of ca. Phytoplasma vitis strains edited by MEGA7 computer program [21]. Sequences were aligned to ca. Phytoplasma vitis FD (♦) sequence (1850 bp DNA, AF176319). Latin names of the infected plant species, the sequence ID#s and the unit of genetic distance (scale 0.01) are indicated

Phytoplasma genome reduction and gene-loss allows time calibration for evolution [4,7,42,52]. Here, we have found divergence of two main groups of Phytoplasma strains at 16S rRNA gene loci (Figure 2).

Several partial but two complete genome sequences of 'ca.' Phytoplasma vitis 'FD' (16SrV-C and 16SrV-D) are available in GenBank [23]. The genome size was found to be 654 223 bp DNA (Table 1) which encodes 498 >506 proteins of the total 548 genes (GCA_023934045.1; CP097583.1; taxid: 131152; GC% 21.5) [53]. The other complete 'ca.' Phytoplasma vitis 'FD' genome shows the same range of genome size with 629 200 bpDNA encoding 507 proteins (GCA_050897995.1; GC% 21.5; taxid: 131152) [27].

The Phytoplasma genomes (Table 1) were found to have extreme low guanine≡cytosine (G≡C) content (21-28 %) compared to the

theoretical 50%, and consequently with high A=T-content. The low GC-content indicates an environmentally responsive 'soft' DNA coupled with adaptive evolution of DNA replication and repair (DRR) [54].

Technically, the phytoplasma genomes were isolated from infected plants, phytoplasma DNA was isolated from infected plants, purified by pulsed-field gel electrophoresis (PFGE) followed by the construction of large-insert libraries and shotgun libraries.

The main α-proteobacteria which supposed to be the donor species of eukaryotic chloroplast and mitochondria might be the species of Rickettsia prowazekii (1 111 523 bpDNA; GC-content 29%; AJ235269.1 and NC_000963); Rickettsia conorii (1 268 755 bpDNA; GC-content 32.5%; AE006914.1 and NC_003103), and further α-proteobacteria of Brucella melitensis, Brucella suis, Caulobacter crescentus, Agrobacterium tumefaciens, Sinorhizobium meliloti, Mesorhizobium loti and Bartonella japonicum [55,56]. These genomes may give the basis to reconstruct plant proto-mitochondrion and proto-chloroplast and to Phytoplasma genomes without 'ca.' prefix.

The plant chloroplasts (cpDNA) and mitochondria (mtDNA) also show low G≡C-content. E.g., the GC%-content of cpDNA Vitis vinifera is about 37.4% [the total length of cpDNA is 159 011 to 163 619 bp sizes] [57,58]. The GC%-content of the huge plant mtDNA genome of Vitis vinifera cultivars and types (all grapes of table, wine, seedless and raisins) varies from 38% to 48% [the total length of V.v. mtDNAs varies 773 298 to 817 446 bp sizes] [which shows >70% similarity to mt-genomes of Nicotiana, Arabidopsis and Zea;] [57,59].

The 'ca.' Phytoplasma Australian grapevine yellows (AusGY) (Table 1) was also found to infect Vitis (and papaya, Carica papaya) [60].

Potential Mobile Units (PMUs) of Phytoplasma Genomes

The phytoplasma genomes were found to contain large 20–75 000 bpDNA long repetitive sequences which are putative transposable elements (i.e., transposons or jumping genes) by sequence orders and were identified as potential mobile units (PMUs) [61]. One of the most studied PMU1-4; is PMU-1 of the Aster Yellows Witches'-Broom phytoplasma (AY-WB) [2]. PMU-1 sequence was found to be flanked by tra5 transposase genes (327 bp each, 172 aa, UniProt A0A0C6FDA5-A0A0C6FDA5_9HYPH) which show similarity to the gene arrangements of IRs (inverted repeats) of retrotransposons [62,63]. However, the cut ('jumping') and reinsertion of PMU-1 into the phytoplasma genome has not been proved [12,61].

Sequences of (pro)phages (phages are viruses which infect bacteria) were also identified in phytoplasma genomes [39]. The genome of 'ca.' Phytoplasma luffae (Luffa aegyptica) strain (NCHU2019; 16SrVIII group) (Table 1) was found to carry two long (75 000 bpDNA) repeat sequences, and about >13 PMUs (most of them with 14-18 kbp DNA length) which showed truncated sequences as they lack of core genes of PMUs (e.g., tmk, dnaB, dnaG, and tra5). The repeats gave large portion (>25%) of 'ca.' Phytoplasma luffae genome [64].

Phytoplasma Solani 'BN' (CaPsol) (CP155828.1) vs. Phytoplasma Vitis 'FD' (CP097583.1)

The 'ca.' Phytoplasma solani (CaPsol) (CP155828.1; subgroup 16SrXII-A, stolbur group) also infects Vitis vinifera with symptoms of 'Bois Noir' (BN) ('black wood') Disease' (Table 1) and show all the symptoms of 'ca.' Phytoplasma vitis FD (CP097583.1) [1,5].

As the two Phytoplasma strains of 'ca.' Phytoplasma vitis 'FD' and 'ca.' Phytoplasma solani 'BN' show nearly the same infection symptoms on grapevine (Vitis vinifera) it was attempted here to find evolutionary linkage between the two host plant species [65]. The result (Figure 3) showed that data of Solanum have a more ancient appearance (>150 MYA) in the evolution compared to Vitis species (>60 MYA) which indicate a more ancient history of 'ca.' Phytoplasma solani 'BN' (CaPsol) compared to 'ca.' Phytoplasma vitis 'FD' (Figure 3).

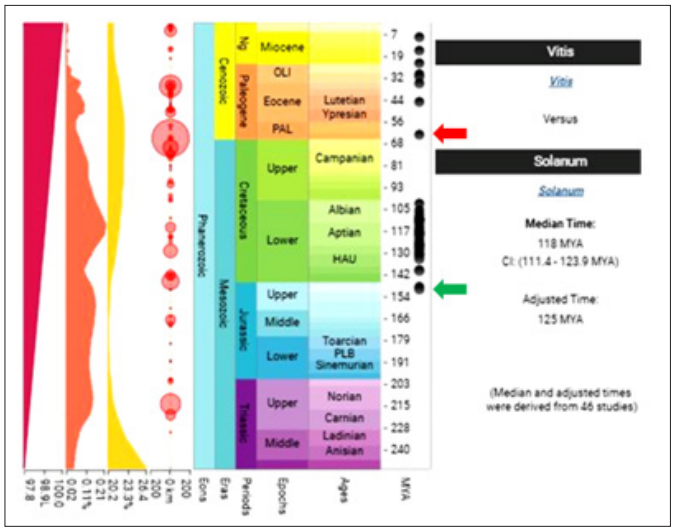

Figure 3: TimeTree of Life5 (TToL5 timetree.org) comparing two Phytoplasma host plants of Vitis and Solanum (46 entries together) with indications of divergence time (MYA), Geological time scale, changes in geological timescale, O_2, CO_2 and solar energy are indicated [52].

Virulence (Syn.: Effector) Proteins of Phytoplasmas

Similar to pathogen bacterial Erwinia ssp. secreted virulence (effector; inducer) proteins the Phytoplasma virulence proteins, e.g., SAP11 of Aster yellows phytoplasma witches'-broom (AY-WB) (Table 1) was found to translocate from phloem tissue sap to plant cell nuclei [61,66-70].

The PHYLLOGEN (PHYL1) protein (90 aa) which cause phyllody (i.e., phyllody-inducing genes of 'ca.' Phytoplasma asteris Onion yellows strain) (Table 1) were also identified ["small proteins with great impact] [39,71].

The effect of TENGU Phytoplasma protein (Tengu-su disease inducer; UniProtKB C0H5W6 / PAM765; 70 aa) (Figure 4) was found to translocate to shoot apical meristem where it down regulates auxin-regulated genes (thus far, it is not clear whether the protein only, or the whole phytoplasma) [22,72]. This is the reason of auxiliary buds sprouts to bushy witches'-broom form [22]. However, several Phytoplasma secreted virulence proteins (>50) have been registered as 'PREDICTED and UNREVIEWED proteins', e.g., from Maize bushy stunt Phytoplasma, Elaeagnus angustifolia witches'-broom Phytoplasma, Catharanthus roseus Aster yellows Phytoplasma, Rapeseed phyllody Phytoplasma, Chrysanthemum coronarium Phytoplasma, Paulownia witches'-broom Phytoplasma, ca. Phytoplasma asteris, and MPEP-jgl1_1 Mulberry yellows witches'-broom Phytoplasma (UniProt.org) [70].

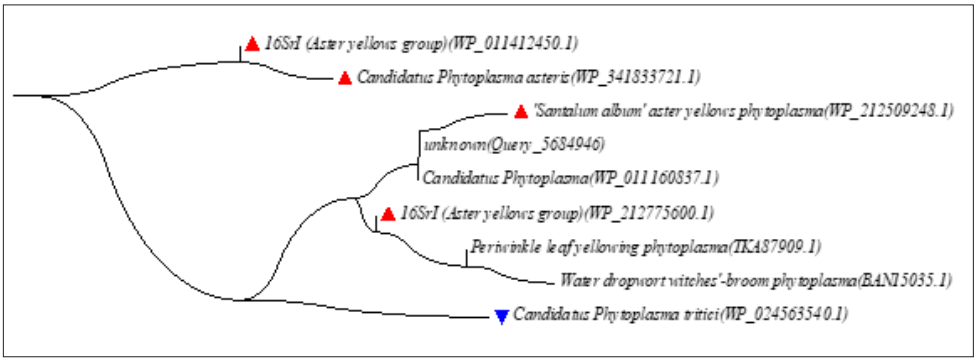

Figure 4: Phylogenetic NJ tree of TENGU proteins of ca. Phytoplasma strains aligned to C0H5W6 sequence [22]. Latin names of infected plant species (Aster, Santalum, and Triticum), sequence ID#s and the unit of genetic distance (scale 0.01) are indicated (16SrI indicates the group of Phytoplasmas).

Phytoplasma Genes Targeted by PCR Primers

In a study of soil bacteria primer pair of 27F and 338R was used. In the analysis of Sass et al. primers of GC357f, containing a 40-bp GC clamp and 907r were used to amplify ca. Phytoplasma genes [73-74].

To increase PCR primer specificity an optimized computer program for targeting Phytoplasma bacterial genes (mopo16S software; Multi-Objective Primer Optimization for 16S experiments under GNU) [75].

Non-specific primers for 16S rDNA-F and 16S rDNA-R were applied in a genome sequencing work of jujube witches'-broom disease (JWB Phytoplasma) (Ziziphus jujuba) [76].

The Phytoplasma ftsH gene (filament temperature-sensitive protein H) (Figure 5) which encodes FTSH intramembrane protein of ATP-dependent metalloprotease complex was found both in prokaryotes and plant organelles (cp and mt) [77]. Three ftsH sequences of ca. Ph. mali (FR863639, FR863637 and FR863645), and five of ca. Ph. ziziphi (WP_121464094.1, WP_121464242.1, WP_121463917.1, WP_121464225.1 and WP_121464146.1) have been sequenced [77]. The ftsH pylogenetic tree showed as reliable as the phylogeny of 16S rRNA genes. GeneBank sequence data of ftsH genes ca. Ph. vitis were analyzed here by BioEdit [78-79] (Figure 5).

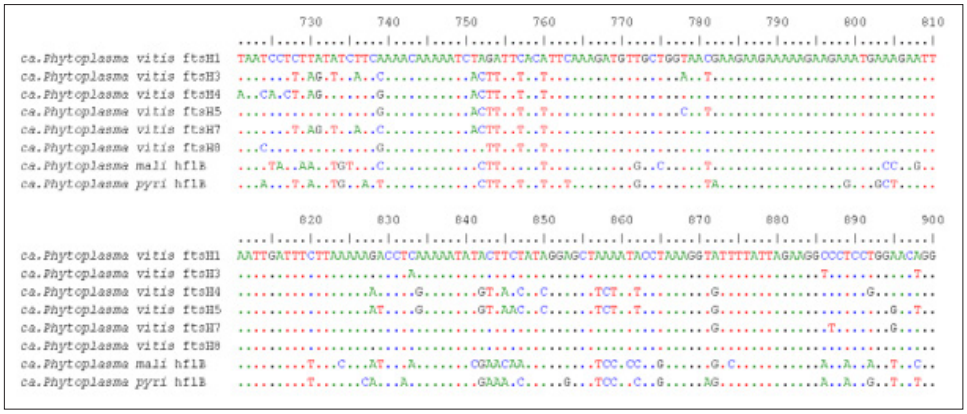

Figure 5: Sequence alignments of ftsH gene sequences (filament temperature-sensitive protein H) of ca. Phytoplasma vitis (720 – 900 nt) (ftsH1,-3,-4,-5,-7,-8) compared to ca. Ph. mali (hf1B, HE819334.1; 1,809 bp) and ca. Ph. pyri (hf1B, HE984352.1; 530 bp, partial) and aligned by computer program BioEdit [79]. The ftsH sequence data of ca. Phytoplasma vitis are ftsH1 (LT999755.1; 1 788 bp); ftsH3 (LT999757.1; 1 593 bp); ftsH4 (LT999758.1; 1 800 bp); ftsH5 (LT999759.1; 1 782 bp); ftsH7 (LT999761.1; 1 584 bp); and ftsH8 (LT999762.1; 2 022 bp DNA).

MLST (multilocus DNA sequence typing) was aimed to reveal the distribution of Paulownia witches'-broom disease (PaWB) (Table 1) in China by analyzing SNPs (single nucleotide polymorphisms) of ten universal housekeeping genes of rp [ribosomal protein gene]; fusA [Fusarium ortholog gene]; secA and secY [secretion protein genes of cell membranes]; dnaK [Hsp70; gene of heat shock protein-70; InterPro; https://www.ebi.ac.uk. IPR013126]; rpoB [DNA-directed RNA polymerase subunit beta, InterPro P0A8V2]; pyrG [CTP synthase, InterPro MF_01227]; gyrB [DNA gyrase subunit-B, InterPro MF_01898]; ipt [Ig-like plexins transcription factors, InterPro IPR002909]; and tuf gene [translation elongation factor Tu gene] [49].

Phytoplasma Genes Targeted by DNA Kits

DNA kits have been developed for Phytoplasma detection. One of them is NIPPON GENE Co. Ltd. 2016 (Code, NE0111) (e-mail: support@nippongene-analysis.com; tel.: 076-451-6548; Univ Tokyo) which targets map gene (methionyl aminopeptidase) of Phytoplasma strains [20].

The DryADD Phytoplasma Universal Detection kit is based on LAMP (Loop-mediated Isothermal Amplification) technology, which uses isothermal DNA amplification with high sequence specificity (https://www.nippongenematerial.com) [80]. LAMP kit was found 1000-fold more sensitive than conventional PCR, RT-PCR, Long-PCR, nested-PCR [20]. The technology was improved by several ways by using Reverse transcription loop-

mediated isothermal amplification (RT-LAMP), multiplex LAMP (mLAMP), colorimetric and fluorescent dyes detection, and real-time monitoring [81-83]. The QUALIPLANTE Inc. Phytoplasma qPCR kit is also on the market (code# UNIPHY24/002; e-mail: info@qualiplante.eu).

The EPHYRA Biosciences Inc. kits use universal nested PCR (https://ephyrabiosciences.com/presta/en/70-phytoplasma) which was developed for the detection of Phytoplasma strains of apple, aster, and elm.

The ZYMOCLEAN GEL DNA recovery Kit, U.S.A. (CA) targets conserved Phytoplasma tuf gene (translation elongation factor Tu gene; EF-Tu; [Tu stands for thermo unstable protein]; 43 kDa in E. coli; in eukaryotes it is eEF1A) (e.g., PP389059.1; direct submission) [84,85].

The FITOLAB Plant Pest Diagnostic and Advisory Ltd, Budapest, Drótos u. 1, 1031, Hungary (tel: 06 1 201 9691; Web: www.fitolab.hu; e-mail: info@fitolab.hu) targets Phytoplasma map gene (methionyl aminopeptidase) [Syn., MetAP; methionyl aminopeptidase; 285–318 aa enzyme, 264 aa in MAP1_Ecoli; EC:3.4.11.18, UniProt.org. P0AE18, which removes the N-terminal methionine from proteins] [86].

Phytoplasma Genomes Complete Genome Sequencing

Phytoplasma genomes were isolated from infected plant tissues, separated and purified from plant (and organelles) DNA by pulsed-field gel electrophoresis, followed by constructing large-insert libraries and shotgun libraries before sequencing (Table 1).

Cross reactions and Possible Misinterpretation of Phytoplasma Identifications

One of the possible reasons of the 'ca.' prefix of Phytoplasma DNA sequences (Table 1, Figure 1,2,4, and 5) is the numerous possible cross reactions in the case of non adequate DNA isolation.

Plant nuclear genomes carry prokaryotic structural 16S rRNA genes as the results of gene transfers from cell organelles to nuclear genome (chloroplasts → mitochondria → nucleus) [57,87]. E.g., the grape mtDNA (773 279 bpDNA, NC_012119) engulfes 68 237 bp cpDNA [which is 42.4 % of the total cpDNA; e.g., of Vitis sylvestris complete cpDNA is 160 928 bp; LC721283], and 8.8% of the mtDNA [59,88]. [The sizes of the huge plant mtDNA mitom varied among Vitis species and cultivars with a range from 817 057 mtDNA bp (of V. vinifera) to 663 157 mtDNA bp (of Vitis rotundifolia) [57]. In Vitis vinifera the length of nuclear–mitochondrial DNA segments (NUMTs) and the nuclear–plastid segments (NUPTs) showed wide DNA sequence ranges among cultivars [57].

In E. coli the 1 541 bpDNA nuclear 16S rRNA gene (J01695.2) was shown to have nine DNA nucleotide variable regions (V1 to V9) along the 69–99, 137–242, 433–497, 576–682, 822–879, 986–1043, 1117–1173, 1243–1294 and 1435–1465 bpDNA stretches, which are also used for Phytoplasma strain identification [89].

A further reason of the possible cross reactions is the abundance of RP (ribosomal proteins) genes, e.g., the genome Arabidopsis thaliana has 409 genes encoding cytosolic, mitochondrial, and chloroplast RPs [90].

Another point is that phytoplasma isolates within a strain share high sequence identity (97.5%) within the 16S rRNA genes [40]. The highly conserved Small and Large SubUnit (S/L-SU) of rRNA genes (Figure 2) of ribosomal proteins (RP) (rps – RP-Small subunit; rpl – RP-Large subunit; rpsl – both RP-Small, and RP-Large subunit) may also show cross reactions [91,92].

The natural ways also produce sources of cross reactions as it was found in the (meta)transcriptomics and RNA-seq analyses indicating virus and microfungi organisms coexisting with Phytoplasma bacteria in the hemolymph of vector insects and in the phloem tissue sap of infected plants [93].

Unkike Spiroplasma citri, which is a culturable Mollicute bacterium, all the ca. Phytoplasmas lack of in vitro aseptic cell culture technology to grow and analyze by biochemical assays, serotyping and antibiotic resistances [14,23]. However, one study of ca. JWB Phytoplasma (Jujube witches'-broom disease of Ziziphus jujuba; Rhamnaceae) strain reports to maintain and propagate Phytoplasma by tissue culture in insect-proof glasshouses [76].

Successful technology uses reservoir weeds, e.g., Garland chrysanthemum (Glebionis coronaria) for the maintenance of onion yellows (OY) phytoplasma strains 'ca.' Ph. asteris; OYW(ild) type; and OYM(ild) pathogenic line [20].

Phytoplasma Disease Control

The most effective controls are the insecticide spraying to control insect vectors, and the removal of infected plants [2]. The use of bactericid antibiotics are prohibited in agriculture (however, not in trunk injection; [94]. Before the regulation, tetracycline treatment was found to be effective against phytoplasma infections [19]. Of a 4-week antibiotic treatment of Phytoplasma infected micropropagated plants in vitro by Tetracycline, Doxycycline, Chloramphenicol, Thiamphenicol, and Rifampicin the Rifampicin treatment was found to be the most successful against Phytoplasma infection [20]. Recently, Phytoplasma resistant Vitis clones and ancient cultivars are suggested to grow (e.g., Georgian local clones) [5,95,96]. Several aseptic methods were also suggested which may eliminate Phytoplasma infections, such as aseptic tissue culture technologies, in vitro embryogenesis, aseptic treatments of infected tissues , the use of natural antimicrobials, in vitro micrografting and shoot tip cultures followed by thermo- or cryotherapy [20,97-102].

Methodology

Phytoplasma gene sequences were downloaded from the NCBI server followed by alignments by ClustalW computer program inbuilt to BioEdit computer program according to Gyulai et al. [79,103-105]. Phylogenetic NJ cladograms were edited by computer program MEGA7-and-12. TimeTree of Life phylograms were edited by computer program TToL5 [52].

Conclusion

All of the Phytoplasma data reviewed and discussed here underline the importance of the analysis of secretional proteins [64]; further serological and plant symptoms detections; the possible application of microdissections for Microgenomics used by Laser Microdissection (LMD) technology (www.leica-microsystems.com); the single cell genomics (SCG) (bigelow.org/scgc); CRISP RNA technology; microflow-chip PCR; microarrays-GeneChip technologies (Affymetrix, Inc.); and to increase Phytoplasma genome specificity to eliminate the prefix 'Candidatus' [33-35,64,109-111].

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