COST Action FA0807
Integrated Management of Phytoplasma Epidemics in Different Crop Systems

Workshop WG1-WG4

Phytoplasma Classification

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COST ACTION FA0807
WORKSHOP WG1-WG4 PROGRAM

Friday July 16, 2010

14:00-18:00 **Phytoplasma Classification**

Convenors: Saskia Hogenhout and Bojan Duduk

14:00-14:30 **Minimum Standards to Name New Species of *Mollicutes*: Deposition of reference Strains**

Daniel R. Brown

14:30-14:50 **Phylogenetic Diversity of Phytoplasma Proteins Suggested by Phylogenetic Analysis Using the Whole Genomic Information**

Kenro Oshima, Hiromi Nishida, Kyoko Sugawara, Nobuko Kojima, Yutaro Neriya, Misako Himeno, Ayaka Hoshi, Yoshiko Ishii, Shigeiuki Kakizawa, Shigetou Namba

14:50-15:10 **Apple Proliferation Taxonomy and Molecular Genetics**

Michael Kube, Bernd Schneider, R. Reinhardt, Eric Seemüller

15:10-15:30 **Combined Actual Gel and Virtual RFLP Analyses for Identification and Classification of Phytoplasmas**

Ing-Ming Lee, Yan Zhao, Wei Wei, and Robert E. Davis

15:30-15:50 **Identification of Phytoplasmas Using DNA Barcodes of Selected Genes**

Nicoletta Contaldo, Olga Makarova, Samanta Paltrinieri, Assunta Bertaccini, Mogens Nicolaisen

15:50-16:10 **Break**

16:10-16:30 **Taxonomy of Phytoplasmas Associated with Coconut Lethal Yellowing-Type Diseases**

Matt Dickinson, Michel Dollet, Nigel Harrison

16:30-16:50 **Insight into the Genetic Diversity among Phytoplasmas in the Stolbur Group**

Fabio Quaglino, Nicoletta Contaldo, Bojan Duduk, Davide Pacifico, Cristina Marzachì, Xavier Foissac, Yan Zhao, Piero A. Bianco, Wei Wei, Paola Casati, Robert E. Davis, Assunta Bertaccini

16:50-17:10 **Genetic Diversity of Flavescence Dorée and Closely Related Phytoplasma Strains of the 16SrV Taxonomic Group in Europe**

Assunta Bertaccini, Elisa Angelini, Patricia Carle, Bojan Duduk, Luisa Filippin, Xavier Foissac, Marta Martini, Samanta Paltrinieri, Pascal Salar, Sylvie Malembic-Maher

17:10-18:00 **Discussion**

Minimum Standards to Name New Species of *Mollicutes*: Deposition of reference Strains

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1. International Union of Microbiological Societies (IUMS) http://www.iums.org
The objectives of the IUMS are to promote the study of microbiological sciences internationally: initiate, facilitate and coordinate research and other scientific activities which involve international cooperation; ensure the discussion and dissemination of the results of international conferences, symposia and meetings and assist in the publication of their reports; represent microbiological sciences in the International Council of Science, and maintain contact with other international organizations. The scientific activities of the IUMS are conducted by the three Divisions: Bacteriology and Applied Microbiology (BAM), Mycology, and Virology, and by six specialist International Committees, eight International Commissions, and two International Federations. Their major activities include the classification and nomenclature of bacteria, fungi and viruses, food microbiology, medical microbiology and diagnostics, culture collections, education, and biological standardization. A meeting of the three divisions occurs every 3 years.
2. World Federation of Culture Collections (WFCC) http://wdcm.nig.ac.jp/wfcc/index.html
The WFCC is one of the Federations within the IUMS. The WFCC is concerned with the collection, authentication, maintenance and distribution of cultures of microorganisms and cultured cells. Its aim is to promote and support the establishment of culture collections and related services, to provide liaison and set up an information network between the collections and their users, to organize workshops and conferences, publications and newsletters and work to ensure the long term perpetuation of important collections. The WFCC World Data Center for Microorganisms is an international database on culture resources worldwide. This data resource is maintained at the National Institute of Genetics in Japan, and currently has records of 537 culture collections from 67 countries. The records contain data on the organization, management, services and scientific interests of the collections. Each of these records is linked to a second record containing the list of species held. The WDCM database forms an important information resource for all microbiological activity and also acts as a focus for data activities among WFCC members.
3. The International Code of Nomenclature of Bacteria (the Bacteriological Code)
At the First International Congress of Microbiology in Paris in 1930, proposals were made for bacteriology to establish its own Code of Nomenclature. In 1936 a draft Code was placed under the authority of an International Committee for Bacteriological Nomenclature (now the International Committee on Systematics of Prokaryotes). The Code was revised most recently in 1990 (International Code of Nomenclature of Bacteria, P. H. A. Sneath, ed., published in 1992 by the American Society for Microbiology) and includes Rules, which are obligatory, and Recommendations, which are guides to good practice. There are sections on how to describe, name and publish on a novel bacterium, and on how to request assistance with nomenclatural problems. It also lists names that have been protected (conserved) and those that must be rejected, and advises on naming of infrasubspecific divisions. [From "A short history of the Bacteriological Code" by P. H. A. Sneath. http://www.the-icsp.org]
4. International Committee on Systematics of Prokaryotes (ICSP) http://www.the-icsp.org
The ICSP is a standing committee of the Division of Bacteriology and Applied Microbiology (BAM) of the IUMS. It oversees the nomenclature of prokaryotes, determines the rules by which prokaryotes are named. The ICSP consists of an Executive Board, a Judicial Commission, and members elected from member societies of the IUMS. In addition, the ICSP currently has 28 Subcommittees that deal with matters relating to the nomenclature and taxonomy of specific groups of prokaryotes, including the Subcommittee on the Taxonomy of *Mollicutes*. The ICSP is also responsible for overseeing the publication of the Bacteriological Code and the International Journal of Systematic and Evolutionary Microbiology (IJSEM) (formerly the International Journal of Systematic Bacteriology).

The Judicial Commission issues opinions concerning taxonomic matters, revisions to the Bacteriological Code, etc. In addition, the Judicial Commission maintains a list of protected names (the Approved Lists of Bacterial Names) together with an official system of registration/indexing of new names and combinations. Only names that have been published in accordance with the Bacteriological Code are considered to have standing in bacterial nomenclature. All new names and combinations validly published appear in the IJSEM. This journal is the official journal of the ICSP and ensures that the system of valid publication of a name or a new combination is also overseen by the ICSP. Valid publication of a new name may be either via an original publication in the IJSEM, or by notification that effective publication of the name has appeared in another journal, via "Validation Lists". Consequently, the ICSP is the sole authority for governing on the implementation of the Bacteriological Code and determining which names are validly published (or may be validly published). [From "The Role of the ICSP (International Committee on Systematics of Prokaryotes) in the Nomenclature and Taxonomy of Prokaryotes" by H. G. Trüper and B. J. Tindall. http://www.the-icsp.org]

5. Rule 30(3b) of the Bacteriological Code

"The description [of a new species] must include the designation of a type strain, and a viable culture of that strain must be deposited in at least two publicly accessible service collections in different countries from which subcultures must be available. The designations allotted to the strain by the culture collections should be quoted in the published description." • Euzéby, J. P. and B. J. Tindall. Status of strains that contravene rules 27(3) and 30 of the Bacteriological Code. Request for an opinion. Int J Syst Evol Microbiol 54 (2004), 293-301.

"After much discussion at recent meetings of the Judicial Commission and ICSP, it was finally decided that some forms of deposit are not widely available and should not serve as type strains. In advance of the publication of the minutes of the Judicial Commission, the wording chosen, which will be changed in Rule 30, was: 'Organisms deposited in such a fashion that access is restricted, such as safe deposits or strains deposited solely for current patent purposes, may not serve as type strains.' The ICSP and its Judicial Commission introduced these rules in order to counter the undesirable trend whereby unreasonable restrictions were placed on the strains or where access was being denied. In this regard, some exceptions may be made, particularly in cases where only a limited number of collections have access to certain technical facilities (e.g. extreme fastidious organisms, extreme barophiles or organisms of Risk Group 3, etc.). In advance of the publication of the minutes of the Judicial Commission the wording which will be added to Rule 30 is: 'Note: In exceptional cases, such as organisms requiring specialized facilities (e.g. Risk Group/Biological Safety Level 3, high pressure, etc.), exceptions may be made to this Rule. Exceptions will be considered on an individual basis, by a committee consisting of the chairman of the ICSP, the chairman of the Judicial Commission and the Editor of the IJSEM. Exceptions will be made known at the time of publication.'

The deposit of strains in at least two different collections, in two different countries, also ensures a system whereby type material is stored in at least two different global locations, ensuring the safe storage of this material. These rules are constantly under review and are intended to uphold an unwritten principle of securing access to type material for furthering the goals of prokaryote systematics." Tindall, B. J., P. Kämpfer, J. P. Euzéby, and A. Oren. Valid publication of names of prokaryotes according to the rules of nomenclature: past history and current practice. Int J Syst Evol Microbiol 56 (2006), 2715-2720.

Phylogenetic Diversity of Phytoplasma Proteins Suggested by Phylogenetic Analysis Using the Whole Genomic Information

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Molecular studies, especially 16S rRNA comparison, have had a widespread impact on the bacterial phylogeny and evolution. However, phylogenetic analysis at the single-gene level may provide only a limited understanding of the relationships and evolutionary history of bacteria, especially closely related species that diverged at almost the same time. In addition, species phylogenies derived from comparisons of different genes do not always concur. This is probably due to lateral gene transfer, saturation for amino acid substitutions, or highly variable rates of evolution of individual genes. Therefore, it is believed that comparative studies based on the complete sequences of bacterial genomes should form the basis for phylogeny.

With the rapid increase in available bacterial whole-genome information, comparison of bacteria at the whole-genome level has proven to be highly useful in microbial phylogenetic research. Here we constructed a phylogenetic tree based on 15 whole genomes of mycoplasmas and the related bacteria. First, 143 orthologous gene families that are shared by all of the 15 bacteria were selected and 143 multiple alignments were generated. Next, a concatenated multiple alignment inferred from the 143 multiple alignments was generated. A total of 43,370 amino acid sites were considered in the neighbor-joining analysis. The phylogenetic tree based on the whole-genomic information indicated that the 15 bacteria were divided into four major groups with 100% bootstrap support, i.e., the *Mycoplasma hyopneumoniae* (Mhy) group, the *M. mycoides* (Mmy) group, the *M. pneumoniae* (Mpn) group, and the *Bacillus*-'*Ca*. Phytoplasma' (BP) group. In the phylogenetic tree, the Mhy group was more closely related to the Mpn group than the Mmy group. The relationships among the Mhy, Mmy, Mpn, and BP groups were supported with 100% in bootstrap analysis. To investigate the contribution of each protein to the whole-genomic phylogenetic tree, we constructed 143 neighbor-joining trees from the 143 protein-sets and classified them. Thirty-nine of the 143 phylogenetic trees had the same type of the topology based on the whole-genome proteins. In contrast, phylogenetic trees of some proteins, such as RpoA, RpoB, RpoC, and RpoD, had the different type of the topology based on the whole-genome proteins or on 16S rRNA gene sequences.

Next, we focused on the phylogenetic relationships between phytoplasmas and other mycoplasmas. Phytoplasmas are plant pathogenic bacteria that are classified into the class *Mollicutes*, and they branched off at an early stage in the evolution of Mollicutes clade. In 104 of the 143 phylogenetic trees of orthologous genes, phytoplasmas were clustered with *Bacillus subtilis*, which agrees with the phylogenetic relationship based on 16S rRNA gene sequences. However, in 39 phylogenetic trees, phytoplasmas were more closely related to mycoplasmas than *B. subtilis*. For example, phytoplasmas were clustered with the *M. hyopneumoniae* group in the phylogenetic tree of ribosomal protein RplL (97% bootstrap support), clustered with the *M. mycoides* group in the phylogenetic tree of DNA gyrase GyrA (42% bootstrap support), and clustered with the *M. pneumoniae* group in the phylogenetic tree of ribosomal protein RplC (52% bootstrap support). These results indicate that the phytoplasma genomes contain some phylogenetically variable genes, probably due to the short evolutionary distance between divergence of mycoplasmas within *Mollicutes* and the branching-off of '*Candidatus* Phytoplasma' genus from the mycoplasmas. Alternatively, some genes might have been integrated into the phytoplasma genome via lateral gene transfer. Our results also suggest that phylogenetic studies using limited sets of genes or proteins are unsuitable for revealing the relationships and evolutionary history of bacteria.

Apple Proliferation Taxonomy and Molecular Genetics

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'*Candidatus* Phytoplasma mali' is causing apple proliferation (AP) disease that severely impairs fruit quality and productivity of the trees. The complete genome sequence of the virulent strain AT was determined and analyzed. Results separate '*Ca*. P. mali' from the other completed phytoplasma genomes by its linear chromosome organization (core and terminal inverted repeat structure), its reduced genome size and gene content. However, genome analysis provided no promising candidates for virulence factors. It also remains unclear if the genome organization is instable due to putative phage integration events and subsequent re-arrangements. Additional information on that is expected from the genome sequence of the largely avirulent `*Ca*. P. mali' strain 1/93. A draft sequence was generated by 454 and clone-based Sanger sequencing reaching a >30-fold coverage. Problems in read length and quality correspond to homopolymer sequences and the low GC content of 22% as it is known for pyrosequencing. Nevertheless, up to 84% percent of all reads could be mapped on the strain AT chromosome. Unmapped reads were assigned to other bacteria, to genome of the tobacco host and to a few genes absent in `*Ca*. P. mali' strain AT, but present in other phytoplasmas. It was unexpected that 87 kb of the strain AT chromosome seems not to be covered by strain 1/93 reads by mapping approaches or reference guided assembly. These preliminary results indicate a genome size below the 602 kb of strain AT. Missing regions could be predicted as phage associated gene clusters. These results demonstrate that the amount of integrated regions was underestimated in the previous study. Apart from these modulations highlighting a rapid evolution, it is clear that the general chromosome organization of '*Ca*. P. mali' is stable. Furthermore, results also highlight that virulence-related genes may be located within the integrated regions present in strain AT, but absent in strain 1/93 showing no symptoms on infected plants. The sequence range to be searched for candidate genes is limited for ongoing studies in consequence.

Combined Actual Gel and Virtual RFLP Analyses for Identification and Classification of Phytoplasmas

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Phytoplasma-specific universal primers developed on the basis of conserved 16S rDNA have enabled researchers to use PCR assays to detect a wide array of phytoplasmas associated with plants and insect vectors. In our laboratory, a nested PCR assay using universal primer pair P1/P7 or P1/16S-SR, followed by primer pair R16F2n/R16R2, is performed routinely to detect known or unknown phytoplasmas from various sources. Initially, detected phytoplasmas are differentiated by the use of selected restriction enzymes for restriction fragment length polymorphism (RFLP) analysis of 16S rDNA (1.25 kb segment) amplified in the nested PCR and identified by comparison of RFLP patterns with those of reference phytoplasmas. In the 1990s, extensive RFLP analyses of PCR- amplified 16S rDNA, from more than 100 phytoplasma strains found in various hosts worldwide, led to the development of a comprehensive phytoplasma classification scheme. Phytoplasmas were differentiated and classified in 16S rRNA (16Sr) groups and subgroups on the basis of their similarity coefficients calculated from collective RFLP patterns of 16S rDNA digested with up to 17 restriction enzymes. The scheme comprised 18 16Sr groups and more than 50 subgroups. Using this scheme, one is able to identify an unknown phytoplasma without having reference strains for comparison. During the last decade, the number of phytoplasma strains reported worldwide has increased exponentially. Without adequate collections of newly reported phytoplasma strains, it becomes difficult to update the classification scheme in a timely manner. This unexpected limitation of using actual gel RFLP analysis for phytoplasma identification and classification has prompted us to seek an alternative approach. Recently, we developed an automated computer-simulated virtual gel RFLP analysis system (iPhyClassifier; http://plantpathology.ba.ars.usda.gov/cgi-bin/resource/iphyclassifier.cgi) that made it possible to exploit the wealth of phytoplasma 16S rDNA sequence data available from GenBank for classification of an unknown phytoplasma, and has allowed us to expand and update the scheme, which now comprises 30 16Sr groups and more than 100 subgroups, making it the most comprehensive phytoplasma classification scheme available. The online iPhyClassifier not only provides rapid identification of an unknown phytoplasma strain, but also generates complete RFLP patterns for strains representing all 16Sr groups and subgroups. The iPhyClassifier embodies several functions: taxonomic assignment, 16Sr group/subgroup classification, virtual gel analysis, virtual gel image, and RFLP pattern comparison. Moreover, the comprehensive classification scheme generated by this system also provides updated RFLP patterns that expand the capacity for classification using actual gel RFLP analysis. While iPhyClassifier expands capacity for phytoplasma classification, it does not obviate the need for verifying key RFLP patterns through actual gel electrophoretic RFLP analysis. To identify putative phytoplasma(s) associated with unknown or newly emerging diseases, we suggest that both actual gel and virtual RFLP analyses should be used in combination. It is generally useful and practical to apply several key restrictions for actual gel electrophoretic RFLP analysis for preliminary identification of putative phytoplasma(s) associated with a given disease, when tens or hundreds of samples need to be analyzed, in order to learn whether a single or multiple phytoplasmas are present among the samples. Once phytoplasmas that are present have been identified at group or subgroup level, representative strains can be selected for sequencing, and their identities can be confirmed and thoroughly documented by virtual RFLP analysis using the iPhyClassifier. In any case, it is important to remember that phytoplasma classification in 16S rRNA groups and subgroups is based solely on results from (actual or virtual) RFLP analysis of rDNA, and not on nucleotide sequence similarities. RFLP analysis also can be applied for finer differentiation of phytoplasma strains using highly variable genetic markers such as ribosomal protein genes or secY gene. In this case, the use of virtual RFLP analysis is preferred over actual gel RFLP analysis because of its higher resolving power in distinguishing DNA restriction fragments of very similar size.

Identification of Phytoplasmas Using DNA Barcodes of Selected Genes

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Barcode regions are used to identify living organisms and the requirements are: polymorphisms to discriminate close relatives; conserved regions for primer design; ideally short 500-700 bp regions. Phytoplasma identification is carried out in a new project funded by EU FP7 to generate barcode sequences from a selected set of genetic regions and for the relevant/quarantine phytoplasmas listed below.

– Elm phloem necrosis (ribosomal subgroup 16SrV-A, strain EY)
– Peach rosette (ribosomal group 16SrIII)
– Peach X (ribosomal subgroup 16SrIII-A, strains CX and WX)
– Peach yellows (ribosomal group 16SrIII)
– Strawberry witches' broom (ribosomal subgroup 16SrI-C)
– Apple proliferation '*Candidatus* Phytoplasma mali' (ribosomal subgroup 16SrX-A, strains AP, AT)
– Apricot chlorotic leafroll '*Candidatus* Phytoplasma prunorum' (rib. subgroup 16SrX-B, ESFY)
– Pear decline '*Candidatus* Phytoplasma pyri' (ribosomal subgroup 16SrX-C, PD)
– Palm lethal yellowing (ribosomal group 16SrIV)
– 'Witches broom' on Citrus '*Candidatus* Phytoplasma aurantifolia' (ribosomal subgroup 16SrII-B)
– Grapevine flavescence doreé (ribosomal subgroups 16SrV-C and 16SrV-D)
– Potato stolbur (ribosomal group 16SrXII)
– Potato purple top wilt (ribosomal groups 16SrI, 16SrVI, 16SrXVIIII)

Phytoplasma 'barcoding' has been performed for many years, particularly using the 16S rDNA, but also other genes such as secY, secA, tuf and ribosomal proteins; however most of these regions span more than 1 kb and/or primers are not generic, which make them impractical for routine barcoding of phytoplasmas. Available sequences of elongation factor Tu (Tuf) and 16S genes were explored for selecting regions suitable for phytoplasma DNA barcoding to develop robust markers of a size that can easily be sequenced. A number of phytoplasma strains (about 60) maintained in periwinkle and field collected were used for PCR amplification with newly developed primers for Tuf and 16S regions and then sequenced. The 5' end of the Tuf and the 5' end of 16S genes were used for barcoding. Sequences of approximately 450 bp for Tuf and 625 bp for the 16S gene were obtained from 60 and 40 phytoplasma strains respectively, belonging to 12 different 16Sr groups. Using these sequences as barcodes it was possible to identify the phytoplasmas into 'Candidatus species' or into 12 of the described 16Sr groups.

Taxonomy of Phytoplasmas Associated with Coconut Lethal Yellowing-Type Diseases

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A major factor that impacts on coconut productivity in Africa, the Caribbean, Florida and Central America, is disease, and in particular, lethal yellowing-type diseases caused by phytoplasmas. Lethal Yellowing (LY) was the first name used to denote the disease in Jamaica, where a second epiphytotic, in which an estimated 3 million palms have been killed, has been underway since the late 1980s. The resistance of both the Malayan Dwarf (MD) ecotype and hybrid MayPan that were introduced to combat the disease following the first epiphytotic in the early 1970s has, for as yet unknown reasons, apparently broken down. Syndromes of LY are characterised by premature fruit drop, blackening of new inflorescences, a progressive yellowing of leaves followed by death of the stem apex leaving bare trucks or 'telephone poles'. In Florida and the Caribbean 16SrIV phytoplasmas are associated with the coconut LY syndrome. Based upon RFLP profiling there are several sub-groups of strains that affect numerous palm species (e.g. date palm, Mexican fan palm and Queen palm) besides coconut. In Africa, coconuts can also be affected by lethal syndromes similar to LY and referred to as Lethal Yellowing-Type Diseases (LYTD). Whilst these have historically been considered as members of 16SrIV, studies based on the 16S-23S rDNA and genes such as secA suggest they should be classified into other groups. In Tanzania and Kenya, the resident strain is referred to as Lethal Disease Tanzania (LDT), which is sufficiently different to those in the Americas to warrant assignment to a new 16Sr group although this has not been formally proposed so far. Strains occurring in the West African countries of Nigeria (Lethal Disease Nigeria LDN, known locally as Awka Disease), Togo (Maladie de Kaïncopé) and Ghana (Cape St Paul wilt disease) are very similar to each other, but sufficiently different from all others according to virtual RFLP analysis to represent yet another distinct group. As such, they have recently been assigned to group 16SrXXII. Surprisingly, the strain identified in Mozambique (East Africa) belongs to the same group as West African isolates, despite the fact that Mozambique borders Tanzania. So far, none of the African strains have been detected in any palm species other than coconut although the syndromes they incite are virtually indistinguishable from those associated with LY in the Caribbean. Recently, phytoplasmas have been implicated with coconut diseases in India (e.g. Kerala wilt), Indonesia (e.g. Kalimantan wilt), Malaysia (Coconut yellow decline) and Sri Lanka (Weligama wilt). However, symptoms indicative of these diseases are less severe than those of LY-type diseases, and the phytoplasmas involved belong to different taxonomic groups (16SrXI and 16SrXIV); two groups commonly associated with diseases of grasses, rice and sugarcane.

Insight into the Genetic Diversity among Phytoplasmas in the Stolbur Group

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Phytoplasmas of the stolbur group infect a wide range of wild and cultivated plants in several areas worldwide. In Europe they are transmitted by polyphagous planthoppers of the Cixiidae family. Based on actual and *in silico* RFLP analyses of 16S rDNA nucleotide sequences subgroups have been described in the stolbur group. In vineyards, grapevine-affecting stolbur phytoplasmas are associated with bois noir (BN) disease and are transmitted by *Hyalesthes obsoletus* Signoret, but in wine-growing areas where *H. obsoletus* is absent, the presence of stolbur phytoplasmas could implies the existence of alternative vectors. Taxonomy of stolbur phytoplasmas on 16S rDNA gene is still poorly studied, and from virtual RFLP analyses there is growing evidence of a discrete amount of variability suggesting delineation of a number of subgroups. Selecting 16Sr DNA sequences from 26 stolbur phytoplasmas infecting diverse plant species such as grapevine, potatoes, corn, and rhododendron in different areas worldwide, and sharing 99% sequence identity, made it possible to distinguish at least 6 different clusters, and many subclusters, based upon nucleotide sequences within the ca. 1,240 bp; actual RFLP patterns confirmed the presence of nine new subgroups in grapevine plants with BN from Northern and Central Italy, Hungary, Serbia and Iran. Key enzymes for distinguishing among these subgroups were *Alu*I, *Bfa*I, *BstU*I, together with *Mbo*II, *Fau*I, *Tsp509*I, and *Hpy188*I. However when compared to its closest relatives, stolbur phytoplasma 16Sr DNA is 97.6% identical to the 16Sr DNA of '*Candidatus* Phytoplasma australiense', 96.5% and 95.3% identical to '*Ca*. P. graminis' and '*Ca*. P. caricae', respectively. Due to its 16S identity with '*Ca*. P. australiense', the designation of stolbur phytoplasma as a '*Candidatus*' species will be not possible without a comparative analysis of non ribosomal *loci*. Biological complexity of stolbur phytoplasmas, indicated by the existence of numerous herbaceous hosts and diverse insect vectors, has stimulated studies on molecular markers of stolbur phytoplasma strains. Characterization of stolbur phytoplasmas in Italian vineyards was performed by *multilocus* sequence analysis of *tuf*, *hlyC*, *trxA-truB*, *cbiQ-glyA*, and *rplS-csdB* genes based on PCR-RFLP assays. *Tuf* gene was selected since two tuf gene sequence variants of stolbur phytoplasmas were found consistently associated with different herbaceous hosts and natural ecologies in Italian vineyards. For each of the other genes it was possible to define two distinct SNP lineages: *hlyC* SNP genetic lineages were consistent with those identified on the basis of *tuf* gene sequences; SNP lineages of *trxA-truB*, *cbiQ-glyA*, and *rplS-csdB* were not consistent with *tuf-hlyC* SNP lineages. Several SNPs of *tuf*, *hlyC*, *trxA-truB*, *cbiQ-glyA*, and *rplS-csdB* genes were positioned within recognition sites of restriction enzymes, and were employed for *multilocus* sequence analyses that grouped the stolbur phytoplasma strains from grapevines in six SNP genetic lineages. Intriguingly, distribution patterns indicated a different prevalence of these SNP lineages in the geographic areas investigated. Furthermore, the finding that the host specificities of stolbur phytoplasma lineages delineated based on *hlyC* and *tuf* genes, points to their possible involvement in the interaction of phytoplasmas with specific hosts. Moreover genotyping of the *SecY* locus revealed 27 stolbur genotypes in the Euro-Mediterranean areas, that formed two major clusters which are congruent with the two tuf groups. The interaction between phytoplasma membrane proteins and host proteins may influence biological and ecological properties of phytoplasmas such as specificity of plants host and insect vectors or symptom induction in plants. Such phytoplasma proteins can be highly variable; therefore their study should present opportunities for further development of phytoplasma genomic markers. Recently, PCR-sequencing and PCR-RFLP genotyping tools were developed targeting the *vmp1* phytoplasma gene, which encodes a variable surface protein. Genotyping of several stolbur isolates from different herbaceous hosts evidenced four genetic variants carrying different *vmp1* RFLP patterns. With the same approach, 12 profiles were identified among stolbur isolates from grapevines, weeds and *H. obsoletus* individuals, clearly indicating a higher variability among French BN strains compared to strains from Italy. Moreover, most of the variability on the *vmp1* gene was associated with type II profiles of *tuf* gene sequences. Interestingly, genetic diversity of stolbur phytoplasmas, determined by 16S rDNA sequence analyses, does not always coincide with that evidenced through the characterization of the other genes studied. Anyway, both the molecular approaches indicated that stolbur phytoplasma strains diversity may be influenced by ecological relationships that alter the composition of populations through strain selection.

Genetic Diversity of Flavescence Dorée and Closely Related Phytoplasma Strains of the 16SrV Taxonomic Group in Europe

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In addition to the grapevine flavescence dorée phytoplasmas (ribosomal subgroups 16SrV-C and -D), other closely related members of group 16SrV infect *Alnus* spp. (16SrV-C), *Clematis vitalba* (16SrV-C), *Spartium junceum* (16SrV-C) and *Rubus* spp. (16SrV-E) in Europe. To investigate which phytoplasmas constitute discrete, species-level taxa, a high number of strains, mainly from grapevine and clematis, were analysed comparing their 16S rDNA and a set of two to five house-keeping genes. Whereas 16Sr DNA identities were well above 97.5% the threshold aimed to distinguish two '*Candidatus* Phytoplasma' species, phylogenetic analysis of the combined sequences of *tuf*, *rplV-rpsC*, *rplF-rplR*, *map* and *uvrB-degV* genes showed that two discrete phylogenetic clusters can clearly be distinguished. The first cluster grouped flavescence dorée (FD), alder yellows (AldY), *Clematis vitalba* and the Palatinate grapevine yellows (PGY) phytoplasmas, and the second cluster was constituted by Rubus stunt phytoplasmas. High genetic diversity was present among phytoplasma strains belonging to the first cluster and several subclusters could be recognized; whereas among Rubus stunt phytoplasmas all analyzed gene sequences resulted highly conserved. Studies based on sequencing, phylogenetic and RFLP analyses of 16S rDNA, *rplV-rpsC* and *rpl15-secY* gene sequences carried out mainly on flavescence dorée phytoplasma strains from Italy, France and Serbia confirmed the presence of genetic variability in phytoplasmas from both flavescence dorée ribosomal subgroups. These subgroups were only differentiated for the presence of a SNP corresponding to a *Taq*I restriction site, however this difference enable to distinguish their geographical and epidemic distribution. Analyses on *rplV-rpsC* and *rpl15-secY* genes reinforced the clear differentiation between flavescence dorée phytoplasma strains of the two subgroups. In particular it was proved the presence of about 10 different flavescence dorée phytoplasma strains in 16SrV-C subgroup, and of 3 different phytoplasma strains in 16SrV-D that were consistently detected in different grapevine varieties and/or in different geographical areas.

In addition to their specificity of described insect vector (*Scaphoideus titanus* and *Oncopsis alni* for phytoplasmas of the first cluster and *Macropsis fuscula* for Rubus stunt phytoplasma in the second cluster), the genomes of the phytoplasma strains belonging to the two distinct clusters above mentioned are differentiated enough at the genomic level to propose two novel putative taxa, '*Candidatus* Phytoplasma caudwellii' for FD, AldY, PGY and *Clematis vitalba* phytoplasmas, and '*Candidatus* Phytoplasma rubi' for rubus stunt phytoplasmas. However, if the rules for the taxonomy of uncultured bacteria were to be strictly applied, this proposition would be not completely acceptable. Indeed, at the 16S rDNA level Rubus stunt phytoplasma can be described as a new species with several specific nucleotide polymorphisms in its 16S rDNA respect to 16SrV-C/D phytoplasmas, in addition to the clear speciation on the five non ribosomal genetic markers. On the contrary, the members of subgroup 16SrV-C/D (FD, AldY, *Clematis vitalba* and PGY phytoplasmas) could not be described as a new species because no specific oligonucleotide polymorphism can be found in their 16S rDNA. Therefore the entire rules as they stand could not be fulfilled for flavescence dorée and closely related phytoplasma isolate, except if a new rule more based on the genomo-species concept was to be established. This is quite relevant, especially considering that FD is a quarantine-regulated pathogen in Europe.

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