New Perspectives on Phytopathogenic Mollicutes

Phytoplasma Identity and Disease Etiology

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ABSTRACT

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Many plant diseases believed to be caused by phytoplasmas were described before phytoplasma groups were delineated through molecular analyses. It is now possible to assess the relationships between phytoplasma identity or classification and specific plant diseases. Data were consistent with the hypothesis of a common ancestral origin of pathogenicity genes in many phytoplasmas and a limited repertoire of plant responses to certain pathogen signals. Observations also were consistent with the hypotheses that the botanical host ranges of some phytoplasmas

reflect specificities in transmission by vectors and vector feeding preferences; phytoplasma-insect vector relationships are keys to understanding evolutionary divergence of phytoplasma lineages; small differences in a highly conserved phytoplasma gene may be regarded as potential indicators of separate gene pools; the reliability of a diagnosis based on symptoms must be learned empirically (i.e., through case study for each syndrome); and some discrete diseases can be ascribed to phytoplasma taxa at the 16S rRNA group level, whereas others are clearly associated with phytoplasma taxa below this level.

Additional keywords: evolution, mycoplasmalike organism, taxonomy.

In plant pathology, as in human and animal medicine, not only pathogens but also the diseases they cause historically have been named largely on the basis of symptoms exhibited by infected hosts. Plant diseases attributed to phytoplasmas are no exception to this practice. For example, many phytoplasma diseases have been classified in general terms, either as decline or virescence diseases. Disease names, such as aster yellows, strawberry multiplier, pear decline, palm lethal yellows, and others, indicate the importance of perceived symptoms in the assignment of common names to diseases. Indeed, common names of pathogens are derived from the names of plant diseases with which they are first associated.

Studies of phytoplasma disease etiology usually have proceeded under the presumption that specific phytoplasmas cause specific diseases. This concept was established even before putative phytoplasma taxa were differentiated and proved correct for some diseases (e.g., apple proliferation, ash yellows, and elm yellows [31, 41, 43]). However, it has become clear that different phytoplasmas can cause apparently identical symptoms in certain plants, including phytoplasmas associated with grapevine yellows, bigbud of tomato, and disorders of periwinkle (*Catharanthus roseus*) (2,6,8, 11,22,38,42). On the other hand, some closely related phytoplasmas can cause distinctly different symptoms in host plants. For example, the syndromes induced by aggressive and nonaggressive strains of ash yellows phytoplasmas in experimentally infected green ash (*Fraxinus pennsylvanica*) differ dramatically (W. A. Sinclair, *unpublished data*). Closely related strains in a single subcluster (subgroup) of the aster yellows genomic cluster (group) can induce either phyllody or small flowers of normal color in infected periwinkle plants. Such observations and the occurrence of

natural infections by mixed phytoplasma populations (2,3,15,24) can hamper disease diagnosis and complicate attribution of a disease to a single phytoplasma taxon.

In this paper, we present a brief assessment of the relationship between classification of phytoplasmas and differentiation of their associated plant diseases. These considerations are important for understanding phytoplasma biology and for successful disease management and regulation of interregional and international shipment of plants that potentially harbor phytoplasmas.

PHENOTYPIC CHARACTERS AND PHYTOPLASMA CLASSIFICATION

Because phytoplasmas cannot yet be isolated in pure culture in vitro, phenotypic characters useful for identification and classification have been difficult to determine. Molecular characterization of conserved genes has presented a partial solution to the problem, enabling the construction of a classification scheme that is independent of phenotypic characters. Several studies of 16S rRNA gene sequences have confirmed the appropriate placement of phytoplasmas within the class *Mollicutes* and have led to recognition of phytoplasmas as a distinct monophyletic clade within the class (16). Phytoplasmas have been classified into more than a dozen groups (Table 1); each group apparently corresponds to at least one species (also listed in Table 1 of Lee et al. [25]). This concept is supported by studies on genes that encode rRNA, ribosomal protein, and the elongation factor Tu (8,15-17,19,21,26,29,36,39, 41,44).

Many plant diseases believed to be caused by phytoplasmas were described before phytoplasma groups were delineated through DNA analyses. It is now possible to assess the relationship between phytoplasma identity or classification and specific plant diseases. The presence, absence, or magnitude of a phenotypic character can be assessed for each phytoplasma strain, and phytoplasma taxa can be compared for characters, such as plant and insect host ranges and propensity to induce particular symptoms,

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such as hyperplasia, phyllody, or witches'-brooms. Schneider and Seemüller (40) assessed the molecular classification of phytoplasmas associated with four symptom types on periwinkle. They found that apart from the virescence-inducing organisms of the aster yellows group (*sensu lato*), there was no consistent relationship between symptom type and phytoplasma group identity. Previously, Lee et al. (22) determined that different disease symptoms could be induced in a host plant by closely related strains classified in the same sub-cluster (subgroup) in the aster yellows phytoplasma genomic cluster.

Because descriptions of organisms cultured *in vitro* are required for naming of species in the class *Mollicutes* and phytoplasmas cannot yet be isolated in culture, phytoplasma lineages are being referred to as "*Candidatus* Phytoplasma species." Two provisional species have been named: *Candidatus* Phytoplasma aurantifolia (47) and *Candidatus* Phytoplasma australiense (8). These designations, as well as others that will be assigned based on analysis of 16S rRNA or other conserved genes, imply that the new taxa are (i) phenotypically distinct and (ii) representative of new lineages. Evidence for the latter is required for publication of a provisional phytoplasma taxon (34,35). However, phylogenetically distinct phytoplasmas already have been found to share some phenotypic characters (e.g., production of a virescence-inducing factor), while displaying other characters that are unique (e.g., host range or vector specificity). Thus, the relationship between phytoplasma classification and specific plant diseases and symptoms appears to be only partially congruent.

Current phytoplasma classification schemes provide for recognition of subgroups within major phytoplasma groups (8,10,15–17,19,26,39,46). Subgroup designation carries the presumption that the subgroup population would be judged phenotypically discrete if its phenotype were known. Presumably, subgroups represent distinct gene pools that have become discrete through evolution after isolation from an ancestral population, perhaps by inhabiting different plant or insect hosts or geographic dispersal of ancestral hosts. Although authorities do not yet agree on criteria for differentiating subgroups, recognition of subgroups seems justified, because some of the subgroups delineated based on molecular criteria apparently have other differentiating properties, including restricted host ranges and limited geographic distribution.

On the other hand, recognition of subgroups based only on minor sequence differences (single-base substitutions) in highly conserved genes can be problematic unless correlated with phenotypic differences. Researchers concerned with phytoplasma classification require criteria for interpreting the significance of small differences in conserved sequences among strains within a group. Single-base substitutions in 16S rDNA may represent (i) intraspecific variability not correlated with any other characteristic that could be used to distinguish species or (ii) indicators of other, as yet undetected, genetic and phenotypic differences or geographic distribution of strains. Fox et al. (12) and Stackebrandt (45) pointed out that in other bacteria, sequence homology >97 to 98% in 16S rRNA genes of an array of strains does not necessarily indicate such strains all belong to the same (species level) taxon. We hypothesize that evolutionary divergence of phytoplasma lineages proceeds through selection for ecological fitness determined by other genes. Because phytoplasma-insect vector relationships and insect vector feeding preferences probably play key roles in opening new ecological niches and providing opportunities for divergent evolution, phytoplasma genes determining fitness for multiplication in and transmission by vectors are probable keys in phytoplasma speciation. Thus, phytoplasma strains sharing sequence identity in 16S rRNA genes may not belong to the same species, and small differences in a highly conserved phytoplasma gene should be regarded as potential indicators of separate gene pools. Because sequence heterogeneity between the two rRNA operons of a phytoplasma has been indicated (9,26,28), it seems advisable to compare equivalent operons when distinguishing closely related phytoplasma strains.

TABLE 1. Selected strains of phytoplasmas classified based on restriction fragment length polymorphism or putative restriction site analysis of 16S rRNA gene sequences

16S rRNA group (subgroup) ^a	Phytoplasma (strain designation)	Source location
16SrI (Aster yellows group)		
I(A)	Tomato big bud (BB)	Arkansas
I(B)	Michigan aster yellows (MIAY)	Michigan
I(C)	Clover phyllody (CPh)	Canada
I(D)	Paulownia witches'-broom (PaWB)	Taiwan
I(E)	Blueberry stunt (BBS1)	Michigan
I(F)	Apricot chlorotic leafroll (ACLR-AY)	Spain
I(K)	Strawberry multiplier (STRAWB 2)	Florida
16SrII (Peanut witches'-broom group)		
II(A)	Peanut witches'-broom (PnWB)	Taiwan
II(B)	Witches'-broom of lime (WBDL), " <i>Candidatus</i> Phytoplasma aurantifolia"	Arabian Peninsula
II(C)	Faba bean phyllody (FBP)	Sudan
II(D)	Sweet potato little leaf (SPLL)	Australia
16SrIII (X-disease group)		
III(A)	X-disease (CX)	Canada
III(B)	Clover yellow edge (CYE)	Canada
III(C)	Pecan bunch (PB)	Georgia
III(D)	Goldenrod yellows (GR1)	New York
III(E)	Spirea stunt (SP1)	New York
III(F)	Milkweed yellows (MW1)	New York
III(G)	Walnut witches'-broom (WWB)	Georgia
III(H)	Poinsettia branch-inducing (PoiBI)	United States
III(I)	Virginia grapevine yellows (VGYIII)	Virginia
16SrIV (Coconut lethal yellows group)		
IV(A)	Coconut lethal yellowing (LY)	Florida
IV(B)	Tanzanian coconut lethal decline (LDT)	Tanzania
16rV (Elm yellows group)		
V(A)	Elm yellows (EY1)	United States
V(B)	Cherry lethal yellows (CLY)	China
V(C)	Flavescence dorée (FD)	France
16SrVI (Clover proliferation group)		
VI(A)	Clover proliferation (CP)	Canada
VI(B)	"Multicipita" phytoplasma	Canada
16SrVII (Ash yellows group)		
VII(A)	Ash yellows (AshY)	New York
16SrVIII (Loofah witches'-broom group)		
VIII(A)	Loofah witches'-broom (LfWB)	Taiwan
16SrIX (Pigeon pea witches'-broom group)		
IX(A)	Pigeon pea witches'-broom (PPWB)	Florida
16SrX (Apple proliferation group)		
X(A)	Apple proliferation (AP)	Germany
X(B)	Apricot chlorotic leafroll (ACLR)	Italy
X(C)	Pear decline (PD)	Italy
X(D)	Spartium witches'-broom (SPAR)	Italy
X(E)	Black alder witches'-broom (BAWB)	Germany
16SrXI (Rice yellow dwarf group)		
XI(A)	Rice yellow dwarf (RYD)	Japan
XI(B)	Sugarcane white leaf (SCWL)	Thailand
XI(C)	Leafhopper-borne (BVK)	Germany
16SrXII (Stolbur group)		
XII(A)	Stolbur (STOL) [formerly group 16SrI, subgroup G]	Serbia
XII(B)	Australian grapevine yellows (AUSGY), " <i>Candidatus</i> Phytoplasma australiense" [formerly group 16SrI, subgroup J]	
16SrXIII (Mexican periwinkle virescence group)		
XIII(A)	Mexican periwinkle virescence (MPV) [formerly group 16SrI, subgroup I]	Mexico
16SrXIV (Bermudagrass white leaf group)		
XIV(A)	Bermudagrass white leaf (BGWL)	Thailand

^a Lee et al. (25), except for new subgroups 16SrI-K, 16SrIII-I, and 16SrVI-B. Location of the source of the representative phytoplasma strain.

At the current stage of phytoplasma classification, it seems worthwhile to take into account small differences in conserved sequences, while reserving judgment about their possible taxonomic significance. This concept recognizes that different subgroups are not necessarily of equivalent taxonomic rank and decisions on taxonomic recognition of narrowly distinguished subgroups ought to await incorporation of other genomic and phenotypic data that could indicate distinct lineages. Nevertheless, provisional designations are needed as aids for disease diagnosis and related regulatory decisions. Consideration of single-base substitutions in highly conserved genes (e.g., 16S rRNA genes) as potential indicators of separate taxa may be a useful approach that will avoid overlooking biological differences or losing molecular distinctions that could later prove to be correlated with biodiversity and significant biological properties that impact disease epidemiology.

Nucleotide sequence data are not available to assess the number of 16S rRNA base substitutions between some closely related subgroups distinguished on the basis of restriction fragment length polymorphism (RFLP) patterns in 16S rDNA, but the occurrence of different RFLP patterns within any of the principal groups is consistent with consideration of subgroups as collectively representing a "16S rRNA species complex," sensu Fox et al. (12). Thus, although each major group may be considered as representative of at least a single phytoplasma species, the subgroups within a group also may be considered as potentially distinct species belonging to the same 16S rRNA species complex. The stolbur and Australian grapevine yellows subgroups, for example, represent distinct lineages and, consequently, have been recognized as separate *Candidatus* Phytoplasma species (8).

PHYTOPLASMA IDENTIFICATION AND DISEASE DELINEATION

The current status of phytoplasma classification and taxonomy raises a practical question: what is the appropriate level of phytoplasma classification at which to differentiate plant pathogens, or what unit of phytoplasma classification should one use to ascribe disease causality? Species is the taxonomic level at which disease causality generally is ascribed in plant pathology. For example, *Pantoea stewartii* causes Stewart's wilt of sweet corn, and *Ralstonia solanacearum* causes bacterial wilt of many plants. On the other hand, advances in fungal genetics have produced several examples of multiple fungal species that cause single diseases (e.g., Dutch elm disease and Verticillium wilt [4,5]). A perfect connection between phytoplasma and disease could be envisioned if each phytoplasma taxon were associated with a discrete disease, i.e., an identifiable set of damaging processes that affects a particular set of plant taxa and presents a particular set of symptoms. However, it appears that the connection is imperfect.

Some discrete diseases can be readily ascribed to phytoplasma taxa at the *Candidatus* species level when equivalent to the 16S rRNA group, whereas others are clearly associated with phytoplasma taxa below this level. For example, ash yellows, lethal yellowing of palms, and rice yellow dwarf, respectively, are associated with three of the major groups (prospective *Candidatus* species) of phytoplasmas. These diseases are discrete in syndrome, spectrum of affected plants, and geographic distribution. However, most phytoplasma diseases that are sufficiently well characterized for consideration appear to be attributable to strain clusters below the 16S rRNA group level, i.e., phytoplasmas belonging to different subgroups often cause different diseases. For example, limited data from the *Prunus* X group (16S rRNA group III) indicate that different subgroups are responsible respectively for peach X-disease, spirea stunt, milkweed yellows, and pecan bunch (13,17). The occurrence of closely related but distinct phytoplasmas in the same geographic region (eastern United States) is consistent with the hypothesis that they have different insect vectors whose respective plant host feeding preferences maintain mu-

tually isolated subgroups. Similarly, in the elm yellows group (16S rRNA group V), it appears that pathogens associated with alder yellows, elm yellows, grapevine flavescence dorée, hemp dogbane yellows, jujube witches'-broom, and perhaps other diseases are distinct based on molecular criteria (14,27,31-33).

There is further evidence that phytoplasmas belonging to different subgroups within a 16S rRNA group can cause different plant diseases. For example, group 16SrXII phytoplasmas in the stolbur subgroup (subgroup A) are acknowledged as the cause of Bois Noir disease of grapevines in Europe and the Mediterranean Region, whereas group 16SrXII phytoplasmas in subgroup B are associated with Australian grapevine yellows disease. The known geographic ranges of these diseases differ from each other, and it seems reasonable to hypothesize that divergent evolution of the pathogens has given rise to distinct phytoplasma species. Comparative analyses of 16S rRNA gene sequences support this hypothesis and the eventual recognition of subgroups 16SrXII-A and 16SrXII-B as separate *Candidatus* Phytoplasma species (8). Although the syndromes caused by these phytoplasmas in grapevines are very similar, the diseases are regarded as different.

SYMPTOMATOLOGY AND PHYTOPLASMAL DISEASE DIAGNOSIS

When a diseased plant is found, one tends to view the syndrome as indicative of a particular disease. Some syndromes induced by specific phytoplasmas may be sufficiently well characterized to permit reliance on symptoms or microscopy for practical diagnosis (e.g., in places where the disease is already known to occur). Examples include ash yellows, elm yellows, and X-disease of chokecherry, in which available evidence indicates only closely related phytoplasma strains are involved.

Disease diagnosis based on symptoms or microscopy in inappropriate circumstances may lead to an erroneous diagnosis and, subsequently, to misdirected efforts at disease management. Precise diagnosis, including pathogen identification, frequently is necessary for effective disease control. Phytoplasmas causing similar syndromes may be spread by different vectors, and effective control may require intervention in the vector cycle. Accurate diagnosis of many phytoplasma syndromes requires specific identification of the pathogen, including syndromes known collectively as grapevine yellows, which are caused by at least three phytoplasmas (1,6,7,30,37,38), bigbud of tomato (11,23,26,42), assorted diseases of periwinkle (18,20,22,26), and all phytoplasma diseases encountered under new circumstances. Specific identification also is needed in cases of different syndromes caused by closely related phytoplasmas. For example, different strains classified in the aster yellows phytoplasma group (16S rRNA group I) may cause identical or different symptoms in the same host species, depending on the strains chosen for comparison (22,26). Understanding the relationship between biological properties and classification based on conserved sequences requires extensive studies of the phytoplasma genome.

CONCLUSIONS

The use of molecular approaches to detect, identify, and classify phytoplasmas has made it possible to (i) determine consistent associations of some phytoplasmas with specific diseases, (ii) recognize some diseases as complexes of several similar disorders caused by different phytoplasmas, and (iii) unveil infections of individual plants by mixed phytoplasma species. Host species and symptoms can indicate the identity of the causal phytoplasma, but the reliability of a diagnosis based solely on symptoms apparently must be learned empirically (i.e., through case study) for each syndrome. Many plant diseases, but very few symptom syndromes, can be differentiated based solely on current phytoplasma classification schemes. Distantly related phytoplasmas often induce very similar

symptoms in a host plant, such as periwinkle. Perhaps this situation prevails because phytoplasmas as a group possess relatively few mechanisms for plant pathogenicity or because phytoplasma attributes that permit parasitism of plants are not highly host-specific. The former would be consistent with the hypothesis of a common ancestral origin of phytoplasma pathogenicity genes and a limited repertoire of plant responses to phytoplasma pathogen signals. The latter would be consistent with the hypothesis that botanical host ranges of some phytoplasmas reflect specificities in transmission by vectors or vector feeding preferences. Additional research is needed to identify linkages between phytoplasma classification and mechanisms of pathogenicity and plant response and to understand the influence of infection by mixed phytoplasma species on the development of disease syndromes.

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