Mixed Infection of Grapevines in Northern Italy by Phytoplasmas Including 16S rRNA RFLP Subgroup 16SrI-B Strains Previously Unreported in This Host

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ABSTRACT

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Grapevine plants of cv. Chardonnay showing symptoms like those of *flavescence dorée* disease in the field in Piemonte, Italy, contained phytoplasmas affiliated with two phylogenetically different 16S rRNA restriction fragment length polymorphism (RFLP) groups. These phytoplasmas were detected and identified by polymerase chain reaction (PCR) amplification of 16S rDNA and by RFLP analysis of amplified DNA as strains belonging to group 16SrI (aster yellows and related phytoplasmas) and group 16SrV (elm yellows and related phytoplasmas). Thirteen of 16 tested plants contained group 16SrI strains. Twelve contained strains belonging to subgroup 16SrI-G (Italian periwinkle virescence and related phytoplasmas), and one contained only a strain belonging to subgroup 16SrI-B (Maryland aster yellows and related phytoplasmas). One plant that contained a subgroup 16SrI-G phytoplasma strains also contained a strain belonging to group 16SrV. Three plants were doubly infected by subgroup 16SrI-G strains and strains belonging to subgroup 16SrI-B. These results indicate susceptibility of grapevines to infection by three distinct phytoplasmas, and reveal for the first time grapevine infection by subgroup 16SrI-B phytoplasmas and mixed infection of single grapevine plants by strains in two different subgroups in group 16SrI.

Additional keywords: molecular detection, mycoplasmalike organism (MLO), *Vitis*

More than 30 years ago, grapevine *flavescence dorée* (FD) disease was described by Levadoux (cited in 28). Subsequently, Caudwell et al. (9) reported transmission of the FD agent from grapevines with symptoms of FD to plants of *Chrysanthemum carinatum* Schousboe and *Vicia faba* L., and transmission of the agent by *Scaphoideus titanus* Ball from *V. faba* to grapevines. Although the cause of FD was attributed to a mycoplasmalike organism (MLO) (8), recently termed phytoplasma (18,30), at present there is no way of knowing the identities of all phytoplasmas transmitted in those studies. The epidemiology of disease termed FD based on symptoms in affected grapevines has been puzzling, probably due in part to the fact that diverse phytoplasmas infect grapevines in regions where *S. titanus* occurs (5,15,26). Recent work presented evidence that the phytoplasma causing FD sensu stricto was related to ash yellows, elm yellows, and western-X phytoplasmas (11) and was a member of 16S rRNA restriction fragment length polymorphism (RFLP) group 16SrV (elm yellows and related phytoplasmas) (26). Although experimental transmission of FD pathogen to several herbaceous hosts has been reported (9), group 16SrV phytoplasmas have not been reported in naturally infected herbaceous plant species in Europe. This observation raises the possibility that some strains transmitted from FD-diseased grapevines to herbaceous hosts in early work (9) may not have been identical to the FD sensu stricto phytoplasma.

Since the earliest work indicated *S. titanus* as a vector of FD (28), this insect has been a major focus in studies of FD (3,4, 10,17,24,25,29). In a recent study, *S. titanus* was proposed as a vector of grapevine yellows disease in New York (23). Although leafhoppers that had fed upon grapevines with symptoms contained antigens that reacted with a polyclonal antiserum prepared against the presumed agent of FD sensu stricto (2,10), identity of any phytoplasma in the insects was not determined, and transmission to grapevines was not demonstrated (23). In a 2-year study of FD in Italy, phytoplasmas were detected in *S. titanus* reared on grapevines with symptoms similar or identical to those of FD. These phytoplasmas were not members of group 16SrV; instead, they were closely related to the group I phytoplasmas of American aster yellows and Italian periwinkle virescence on the basis of results from dot hybridizations using cloned phytoplasma DNA probes (4). Since FD has been defined as a grapevine yellows disease caused by a phytoplasma transmitted by *S. titanus* (2,10), and since FD and other grapevine yellows diseases can exhibit apparently identical symptoms (7), it is important to clarify the identities of phytoplasmas infecting plants with these symptoms. Here we confirm mixed infection of grapevine in Italy by group 16SrI and 16SrV phytoplasmas, and we report for the first time susceptibility of grapevines to infection by subgroup 16SrI-B phytoplasmas and mixed infection of single grapevine plants by strains in subgroups 16SrI-B and 16SrI-G in areas where *S. titanus* has been reported (32).

MATERIALS AND METHODS

Plant samples. During autumn of 1993 and 1994 in the Piemonte region of northwestern Italy, leaf samples were collected from shoots exhibiting symptoms on 16 diseased plants of grapevine (*Vitis vinifera* L. 'Chardonnay') with symptoms described (8,9,28) for FD disease. Known control (reference) phytoplasmas were maintained in greenhouse-grown plants of periwinkle (*Catharanthus roseus* (L.) G. Don). These phytoplasmas included strains associated with Maryland aster yellows (AY) (22) and Italian periwinkle virescence (IPVR) (14,16) field collected in Beltsville, MD, and Emilia-Romagna (Italy), respectively, elm yellows (EY) kindly provided by W. A. Sinclair, Cornell University, Ithaca, NY, and Canada X disease (CX) and clover phyllody (CPh) kindly provided by L. N. Chiykowski, Plant Research Center, Agriculture Canada, Ottawa, Ontario. Table 1 gives the 16S rRNA RFLP group and subgroup affiliations and source locations of these strains as well as those of selected examples of related phytoplasmas. Periwinkle and grapevine plants grown from seed were used as healthy controls.

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Nucleic acid extraction and PCR conditions. Total nucleic acid for use as template in polymerase chain reaction (PCR) was extracted from approximately 2 g of freshly stripped midribs from periwinkle and grapevine plants according to a previously described procedure (15,20). Tubes with the reaction mixture devoid of DNA templates were included in each experiment as negative controls.

The following four pairs of oligonucleotides were used as primers in PCR to amplify 16S rDNA: phytoplasma-universal primer pair R16F2/R2 (F2/R2) (sequence according to positions given in reference 22), phytoplasma-universal primer pair R16F1/R0 (F1/R0), group 16SrI-specific primer pair R16(I)R1/F1 [=R16(I)], group 16SrV-specific R16(V)R1/F1 [=R16(V)] (21,22). These primer pairs were used in experiments of direct (non-nested) and/or nested PCR. For direct PCR, nucleic acid samples were diluted in sterile deionized water to give a final concentration of 20 ng/µl and used in PCR reaction as described (21). In nested PCR, DNA amplification was first carried out in reaction mixtures containing template consisting of total nucleic acid extract and primer pair R16F1/R0 or R16F2/R2; the product (completed reaction mixture) from this first PCR was then diluted 1:50, or 1:160 where specified, and used as template for a second (nested) PCR containing group-specific primer pair R16(I)F1/R1 or R16(V)F1/R1 (21). All the PCR assays were run for 35 cycles under parameters already described (21).

RFLP analyses of PCR products. Phytoplasma 16S rDNA sequences amplified in nested PCR using primer pair R16F2/R2 or R16(I)F1/R1 were analyzed by restriction endonuclease digestion (22, 27). Five microliters of each PCR product was digested separately with three selected restriction endonucleases, *Alu*I and *Hha*I (Life Technologies, Gaithersburg, MD), and *Mse*I (New England Biolabs, Beverly, MA). The digestion products were then analyzed by electrophoresis through a 5% polyacrylamide gel followed by treatment with ethidium bromide and visualization of DNA bands using a UV transilluminator. Repeated tests were carried out in an attempt to detect and identify phytoplasmas in all diseased grapevine samples; samples not represented in figures from gel electrophoretic analyses are those in which phytoplasmas were not detected. Determination and nomenclature of 16S rRNA RFLP groups are according to Lee et al. (22) as amended to include subgroup 16SrI-G (31). Affiliation of a phytoplasma with group 16SrI subgroup I-G was based on identity of RFLP patterns of amplified DNA with those of DNA from IPVR phytoplasma, since those of DNA from IPVR were identical to RFLP patterns for phytoplasmas in subgroup I-G (R. E. Davis and A. Bertaccini, unpublished).

RESULTS

Detection and identification of phytoplasmas in grapevines. Based on amplification of DNA in PCR primed by group-specific primer pairs, phytoplasmas were detected in 13 of the 16 grapevine plants sampled. Repeated tests gave the same results. A group 16SrV phytoplasma was detected in only one plant, 11G (Fig. 1A), whereas phytoplasmas belonging to group 16SrI (aster yellows and related phytoplasmas) were detected in most of the diseased grapevine plants (Fig. 1B). No phytoplasmas were detected in healthy control plants or in three grapevines with symptoms. No amplification was detected in the samples devoid of template DNA. The assignment of the group 16SrI phytoplasmas to different subgroups was accomplished by RFLP analyses of amplified DNA (Table 2). RFLP patterns of most DNA fragments amplified in PCR containing templates from diseased grapevines were those characteristic of either group 16SrI, subgroup I-B, or group 16SrI, subgroup I-G, phytoplasmas (Figs. 2 and 3). In particular, subgroup 16SrI-G phytoplasmas were present in all except one (17G) of the 13 phytoplasma-positive grapevine plants in which phytoplasmas were detected (Table 2); these plants included the one (11G) in which the subgroup 16SrI-G phytoplasma was present in mixed infection with a phytoplasma belonging to group 16SrV. A phytoplasma belonging to subgroup I-B was detected in

Table 1. Geographical location of source and 16S rRNA restriction fragment length polymorphism (RFLP) group and subgroup affiliations of reference phytoplasmas used in this study and of selected examples of related strains[a]

	16S rRNA		Related phytoplasma(s)	
Reference phytoplasma (source)	Group	Subgroup	Strain	Source
Maryland aster yellows (U.S.)	16SrI	I-B	Hydrangea phyllody	Italy
Clover phyllody (Canada)	16SrI	I-C		
Italian periwinkle virescence (Italy)	16SrI	I-G	Stolbur	Europe
			Bois noir	Europe
Elm yellows (U.S.)	16SrV		*Flavescence dorée*	Europe
Canada X-disease (Canada)	16SrIII	III-A	Western X-disease	U.S.

[a] Affiliations of phytoplasmas as previously determined (22,26; R. E. Davis, unpublished; R.E. Davis and A. Bertaccini, unpublished). Related phytoplasmas are examples and do not constitute an exhaustive list.

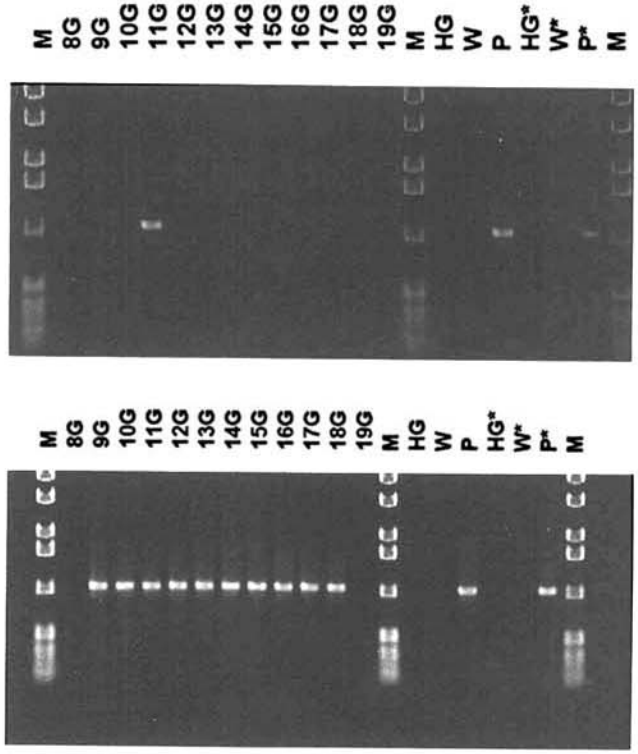

Fig. 1. Nested polymerase chain reaction (PCR) amplification of phytoplasma 16S rDNA from naturally diseased cv. Chardonnay grapevines with symptoms characteristic of *flavescence dorée*. The first amplification was obtained with primers R16F2/R2, and the results shown in the figure are from the nested PCR using primer pair R16(V) (upper) or R16(I) (lower). Lanes: M, 1-kb DNA ladder (Life Technologies, Gaithersburg, MD); from 8G through 19G, symptomatic grapevines; HG, healthy grapevine; W, sample devoid of DNA template; P, positive control (in upper, Elm yellows and in lower, Maryland aster yellows phytoplasma); *, product from direct (non-nested) PCR.

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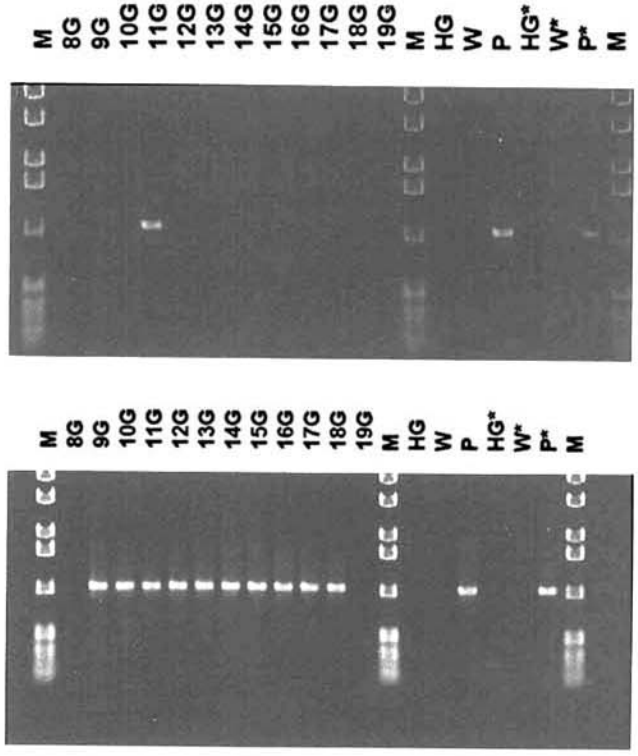

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one plant (17G) in which no other phytoplasma was detected (Fig. 2).

Phytoplasma-specific DNA amplified from template derived from three grapevine plants (1G, 3G, 16G) yielded identical RFLP patterns that were distinct from those of DNAs amplified from any control phytoplasma. (Patterns from two plants are shown in Figure 3.) Instead, each pattern was a composite pattern containing DNA bands characteristic of the RFLPs of DNA from group 16SrI subgroup I-B and subgroup I-G phytoplasmas, indicating mixed infection by strains in both subgroups.

DISCUSSION

In this paper we present evidence that Chardonnay grapevines with symptoms like those of FD in Piemonte can be infected by three distinct phytoplasmas. To our knowledge, this work is the first to demonstrate infection of grapevines by strains in subgroup I-B of 16S rRNA RFLP group 16SrI (aster yellows and related phytoplasmas). Subgroup I-B has been shown to be associated with American aster yellows disease (22) and, recently, subgroup I-B strains were reported in apricot, nectarine, and Japanese plum in Italy (19). Previous authors concluded that the presumed pathogen of FD sensu stricto was related to several different phytoplasmas including those in a group (designated group IV by Ahrens and Seemüller) (1), which contains ash yellows and elm yellows phytoplasmas (1,11–13). Other work showed that FD phytoplasma was a member of the 16S rRNA RFLP group 16SrV (elm yellows and related phytoplasmas), while ash yellows phytoplasma represents a separate group (26). We detected a group 16SrV phytoplasma in only one of 16 grapevine plants with symptoms like those of FD, in spite of the fact that the plants were from a region where *S. titanus*, the reported vector of FD pathogen (32), occurs. In contrast, strains closely related to IPVR phytoplasma, which represents a separate subgroup (I-G) in group 16SrI (R. E. Davis and A. Bertaccini, unpublished), were detected in most plants studied. It appears that the yellows disease of grapevine in Piemonte can be associated with the presence of varied phytoplasmas. Our finding that only one plant with FD-like symptoms contained a group 16SrV phytoplasma is consistent with the possibility that subgroup 16SrI-G strains were responsible for the symptoms observed in most of the plants. This possibility raises questions about the accuracy of a previous suggestion (2) that grapevine yellows diseases be called FD if they occur where *S. titanus* is found. It is clear from the present and separate work (5,6) that other grapevine yellows diseases also occur within the geographical range of FD and *S. titanus* in Europe. Results of the present study indicate that subgroup 16SrI-G phytoplasmas should be pursued as one of the causes of grapevine yellows in Piemonte.

The grapevine yellows disease termed *bois noir* (BN) has been distinguished

Table 2. Results from restriction fragment length polymorphism (RFLP) analysis of amplified DNA products from direct (non-nested) and nested polymerase chain reactions (PCR) using phytoplasma universal and group I-specific primers and template DNA derived from naturally diseased grapevine plants in which phytoplasmas were detected

	Identification of phytoplasmas detected[a]	
Grapevine plant	**Subgroup 16SrI-B[b]**	**Subgroup 16SrI-G[b]**
1G	+ (b)	+ (b)
3G	+ (b)	+ (b)
4G	– (b)	+ (b)
9G	– (a,b)	+ (a,b)
10G	– (a,b)	+ (a,b)
11G	– (a,b)	+ (a,b)
12G	– (a,b)	+ (a,b)
13G	– (a,b)	+ (a,b)
14G	– (a,b)	+ (a,b)
15G	– (a,b)	+ (a,b)
16G	+ (a)	+ (a)
17G	+ (a,b)	– (a,b)
18G	– (a)	+ (a)

[a] +, presence of phytoplasma subgroup indicated; –, indicated phytoplasma subgroup not detected.
[b] Subgroup affiliation based on (a) RFLP pattern of DNA amplified in direct (non-nested) PCR using universal primer pair R16F2/R2 or (b) RFLP pattern of DNA amplified in nested PCR primed by group 16SrI-specific primer pair R16(I)F1/R1. Maryland aster yellows (AY) phytoplasma was used as known control in detection and identification of subgroup 16SrI-B strains. Italian periwinkle virescence (IPVR) phytoplasma, a member of a distinct group 16SrI subgroup (subgroup I-G) that also contains European tomato stolbur disease phytoplasma (R. E. Davis and A. Bertaccini, unpublished), was used as a known control in detection and identification of subgroup 16SrI-G strains.

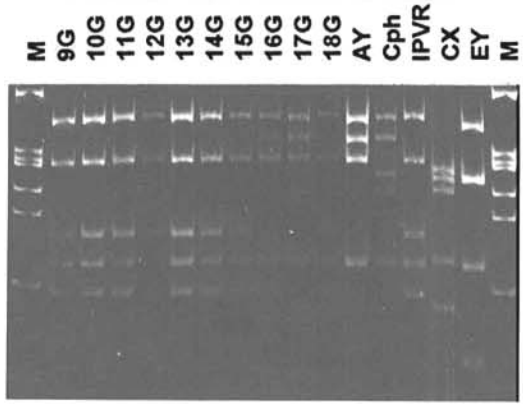

Fig. 2. Restriction fragment length polymorphism (RFLP) analyses of DNA amplified in nested polymerase chain reactions using primer pair R16F1/R0 followed by primer pair R16F2/R2 from template DNA extracted from naturally diseased cv. Chardonnay grapevines with symptoms characteristic of *flavescence dorée* or from plants of periwinkle containing control phytoplasma strains. Amplified DNA was digested with *Mse*I. Lanes: M, phiX174 RF I DNA *Hae*III digest, fragment sizes in base pairs from top to bottom are 1,078, 872, 603, 310, 281, 271, 234, and 194; samples 9G through 18G, grapevine samples; AY, Maryland aster yellows; CPh, clover phyllody; IPVR, Italian periwinkle virescence; CX, peach X disease from Canada; EY, elm yellows.

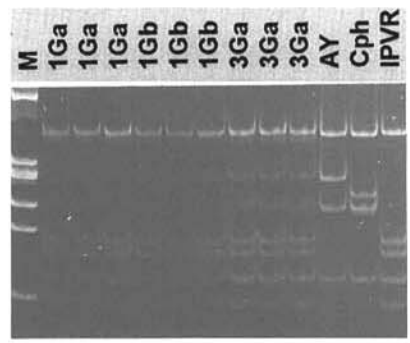

Fig. 3. Restriction fragment length polymorphism (RFLP) analyses of nested polymerase chain reaction products obtained using primer pair R16F2/R2 followed by primer pair R16(I) specific for group I phytoplasmas. Template in initial reaction consisted of DNA extracted from naturally diseased cv. Chardonnay grapevines with symptoms characteristic of *flavescence dorée*. Template in nested reaction consisted of product from initial reaction. Amplified DNA was digested with *Mse*I. Lanes: M, phiX174 RF I DNA *Hae*III digest, fragment sizes in base pairs from top to bottom are 310, 281, 271, 234, 194, and 118; 1G and 3G, diseased grapevine samples (a: template for nested reaction was diluted 1:50; b: template for nested reaction was diluted 1: 160); AY, Maryland aster yellows; CPh, clover phyllody; IPVR, Italian periwinkle virescence.