

## Flavescence dorée in France and Italy - Occurrence of closely related phytoplasma isolates and their near relationships to Palatinate grapevine yellows and an alder yellows phytoplasma

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### Summary

Grapevine yellows (GY) are diseases of *Vitis vinifera* caused by phytoplasmas. On the basis of DNA analysis, it is possible to distinguish different groups and subgroups among grapevine phytoplasmas. Flavescence dorée (FD), the most serious problem in European vineyards, is caused by a phytoplasma which belongs to the elm yellows group (EY or 16SrV). Differences between 7 phytoplasma isolates in this group, found until now in French, Italian and German grapevines, and 4 phytoplasmas in the same group, isolated mainly from elm and alder in Europe and America, were investigated. These 11 EY-group isolates plus 3 non-EY phytoplasmas, were compared by PCR-RFLP analyses of two different DNA fragments using 8 restriction enzymes. Two French and two Italian grapevine isolates, classified as FD, appeared to be closely related and were also closer to an Italian alder phytoplasma. One French and one Italian FD isolate always showed the same restriction pattern. On the opposite, the three German grapevine isolates related to alder phytoplasmas in Germany appeared to be closer to the two elm phytoplasmas from America and Europe.

**Key words:** phytoplasma, grapevine yellows, Flavescence dorée, elm yellows, PCR, RFLP.

### Introduction

The generic name of Grapevine yellows (GY) has been given to grapevine diseases associated to phytoplasmas. Phytoplasmas are non-cultivable plant pathogenic mollicutes that have been classified on the basis of direct sequencing or RFLP analyses of their 16S rDNA (LEE *et al.* 1998; SEEMÜLLER *et al.* 1998). The subclades identified in this way are considered to represent distinct species of phytoplasmas (ICSB 1997). All GY diseases show similar symptoms in *Vitis vinifera*, but it was demonstrated, on the basis of DNA analyses, that different clusters of phytoplasmas are associated with these diseases (DAIRE *et al.* 1993, 1997 a; BERTACCINI *et al.* 1995). The most important in Europe are Flavescence dorée (FD) and Bois Noir (BN) or Vergilbungskrankheit (VK) (GÄRTEL 1965; CAUDWELL *et al.* 1971 a, b; DAIRE *et al.* 1993, 1997 a; MAIXNER *et al.* 1994, 1997; BERTACCINI *et al.* 1995;

LAVIÑA *et al.* 1995; MARCONE *et al.* 1996; BATLLE *et al.* 1997; DAVIS *et al.* 1997). Phytoplasmas associated to BN and VK belong to the stolbur (STOL) group (16SrXII-A) and are transmitted by the occasional grapevine-feeder Cixiidae *Hyalesthes obsoletus* Sign. (MAIXNER 1994; SFORZA *et al.* 1998). On the other hand, the etiological agent of FD belongs to the elm yellows (EY) or 16SrV group (DAIRE *et al.* 1992, 1993, 1997 a; BERTACCINI *et al.* 1995). It is specifically transmitted by *Scaphoideus titanus* Ball., a Cicadellidae of American origin (VIDANO *et al.* 1966; MAIXNER *et al.* 1993) which spends its whole life cycle on *Vitis* spp. and disseminates FD in an epidemical manner. It is important to specify that the name of FD *sensu stricto* has been reserved only for GY diseases transmitted by the latter leafhopper (BOVEY and MARTELLI 1992). New outburst of the disease in distant regions is mainly attributed to propagation by latently infected plant material planted in areas where *S. titanus* is present (CAUDWELL *et al.* 1994; BOUDON-PADIEU and MAIXNER 1998).

Previous studies have demonstrated that different phytoplasma isolates in the EY group can be associated with FD or FD-like diseases. In France, different FD phytoplasmas have been isolated: FD70, FD88 and FD92 (BOUDON-PADIEU *et al.* 1987, 1990; DAIRE *et al.* 1992, 1997 b; CLAIR *et al.* 2000). In Italy, two different isolates have been found in grapevines (DAIRE *et al.* 1997 b) and further designated FD-C and FD-D (BERTACCINI *et al.* 1997; MARTINI *et al.* 1999). In Germany, in the Palatinate region, EY-group phytoplasmas have been detected in grapevine (MAIXNER *et al.* 1995) and three different types were described and called PGY-A, PGY-B and PGY-C (PGY = Palatinate Grapevine Yellows) (DAIRE *et al.* 1997b; MAIXNER and REINERT 1999). They were shown to be transmitted from the black alder, *Alnus glutinosa* to alder and grapevine by the alder-feeding Hemiptera *Oncopsis alni* (MAIXNER and REINERT 1999; MAIXNER *et al.* 2000). Transmission assays of PGY isolates from naturally infected cv. Scheurebe to healthy grapevine seedlings with *S. titanus*, which was never found in Palatinate, failed until now (BOUDON-PADIEU and MAIXNER, unpubl.).

It is important to look at the epidemiology of these diseases. PGY are not epidemic (REINERT 1999; REINERT and MAIXNER 2000), probably because their natural vector feeds on grapevine only occasionally. On the other hand, FD is very epidemic because *S. titanus* is present on vines in high population densities, mainly in some regions of France and

Italy. In recent years in France, only FD92 has been detected in vineyards, while FD70, isolated in the seventies, has not been found in French grapevine fields since 1987 (DAIRE *et al.* 1997 b; BOUDON-PADIEU, unpubl.). In Italy, FD-C is present in Piemonte, Lombardia and Veneto (MARTINI *et al.* 1999; ANGELINI *et al.* unpubl.), while FD-D has been found so far only in Veneto (MARTINI *et al.* 1999), Friuli Venezia Giulia, (ANGELINI, unpubl.) and in Emilia (BERTACCINI *et al.* 2000).

The present study compares the different EY-group grapevine phytoplasma isolates detected in France, Italy and Germany and related phytoplasmas hosted by other reservoirs than *Vitis* spp., in order to obtain more information on genetic relationships of phytoplasma isolates transmitted or not transmitted by *S. titanus*. The final objectives of such investigations would be to develop tools to evaluate the risk that new phytoplasmas erratically transmitted to grapevine could be further spread epidemically by *S. titanus*, which lives in a much wider geographic area than the present extension of FD epidemic (BOUDON-PADIEU 2000).

### Material and Methods

**Source of phytoplasmas:** In this study 14 phytoplasma isolates were used. Two French FD isolates were analyzed: FD70, maintained in periwinkle, and FD92, maintained in broadbean. Both isolates have been transmitted from grapevine to broadbean (*Vicia faba* L.) in 1970 (CAUDWELL *et al.* 1970) and 1992 respectively and maintained in broadbean through serial transmission by the leafhopper *Euscelidius variegatus* (CAUDWELL *et al.* 1972); in 1991 FD70 has been transmitted with *E. variegatus* from broadbean to periwinkle (*Catharanthus roseus* L.) and maintained since in periwinkle by serial grafting. The two Italian FD isolates, FD-C and FD-D, were obtained from naturally infected grapevine leaves in Italian vineyards. The three German isolates, PGY-A, PGY-B and PGY-C, were kindly provided by M. MAIXNER (Institut für Pflanzenschutz im Weinbau, BBA, Bernkastel-Kues, Germany) as leaf tissues from naturally infected field-grown vines. Other EY-group phytoplasma isolates, maintained in periwinkle, were included: EY1, elm yellows (isolated by W.A. SINCLAIR, New York), and ULW, elm witches' broom (isolated by G. MORVAN, France), both belonging to the 16SrV-A cluster; HD1, hemp dogbane yellows (isolated by H.M. GRIFFITHS, New York); ALY, Italian alder yellows (isolated by C. MARCONE, Napoli); STOLD,

"stolbur" of tomato (provided by M.T COUSIN, France and originating from tomato in Brazil) previously classified into the EY group (BOUDON-PADIEU *et al.* 1996). As control, phytoplasma isolates belonging to different phylogenetic groups in the classifications by SEEMÜLLER *et al.* (1998) and LEE *et al.* (1998), transmitted and maintained in periwinkle by serial grafting, were utilized: AAY, American aster yellows (Florida) from aster yellows group (16SrI-B), and STOLC, stolbur of tomato (France) from stolbur group (16SrXII-A).

**DNA extraction:** Nucleic acids were extracted from fresh leaf and vein tissues of broadbean, periwinkle or grapevine using the CTAB (cetyltrimethylammonium bromide) extraction described for periwinkle by DAIRE *et al.* (1997 b), with some modifications: 1 g of tissue was ground in 7 ml of extraction buffer (3 % CTAB, 100 mM Tris-HCl pH 8, 10 mM EDTA, 1.4 M NaCl, 0.1 % 2-mercaptoethanol). The suspension (1 ml) was transferred to an Eppendorf tube and incubated for 20 min at 65 °C. After extraction with an equal volume of chloroform, the aqueous phase was recovered and the nucleic acids precipitated with an equal volume of isopropanol and collected by centrifugation. The DNA pellet was washed with 70 % ethanol, dried and dissolved in 150 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.6). The Italian grapevine samples were extracted following a different procedure (PRINCE *et al.* 1993).

**Primers for PCR amplification of phytoplasma DNA:** rDNA was amplified in a nested-PCR procedure with universal primers for phytoplasmas. The first pair of primers was P1 (DENG and HIRUKI 1991) and P7 (SMART *et al.* 1996) and the second pair was 16r758f (GIBB *et al.* 1995) and M23Sr (PADOVAN *et al.* 1995). The final 16r758f/M23Sr product extends from position 758 in the 16S rRNA gene through the intergenic spacer until the beginning of the 23S rRNA gene; its size is about 1050 bp.

For specific amplification of the FD9 non-ribosomal DNA fragment of EY-group phytoplasmas, 3 primers, namely FD9f2, FD9f3 and FD9r2, in addition to FD9f/r primers designed by DAIRE *et al.* (1997 b), were constructed using the aligned FD9 sequence in 5 EY-group isolates obtained by REINERT (1999). The sequences of all the 5 FD9 primers are given in Tab. 1. In a nested-procedure, the first pair of primers used was FD9f2/r and the second pair was FD9f3/r2. The expected size of the final FD9f3/FD9r2 product is about 1150 bp (CLAIR *et al.* 2000).

**DNA amplification:** DNA amplification was performed in 25 µl total reaction volume in a Biometra T3 thermocycler. The reaction mixture contained as template

Table 1  
Sequences of primers constructed on both ends of FD9 DNA fragment

Primer name	Sequence	bp	Reference
FD9f	5'-GAATTAGAACTGTTTGAAGACG-3'	22	DAIRE <i>et al.</i> 1997 b
FD9f2	5'-GCTAAAGGTGATTAAAC-3'	17	This paper
FD9f3	5'-GGTAGTTTTATATGACAAG-3'	19	This paper
FD9r	5'-TTTGCTTTCATATCTTGTATCG-3'	22	DAIRE <i>et al.</i> 1997 b
FD9r2	5'-GACTAGTCCCGCCAAAAG-3'	18	This paper

1 µl of extracted DNA or of the diluted (1:50) first PCR product, 0.25 mM each dNTP, 0.5 µM each primer, 1.5 U Appligene *Taq* polymerase and the buffer supplied with the enzyme. For amplification with primers FD9, 1 mM MgCl<sub>2</sub> was added. Reaction mixture with deionized water in place of plant nucleic acid was used as negative control.

With primer pair P1/P7, the following conditions were used in 35-cycle PCR: denaturation at 92 °C for 45 s (2 min 15 s for the first cycle), annealing at 57 °C for 45 s and primer extension at 72 °C for 1 min 45 s. With primer pair 16r758f/M23Sr, conditions for 35-cycle PCR were denaturation at 92 °C for 1 min (2 min for the first cycle), annealing at 50 °C for 2 min and primer extension at 72 °C for 3 min (Schaff *et al.* 1992). With primer pair FD9f2/r, 35-cycle PCR was performed according to the following steps: denaturation at 92 °C for 30 s (1 min 30 s for the first cycle), annealing at 47 °C for 30 s and primer extension at 72 °C for 1 min 15 s. With primer pair FD9f3/r2, 40-cycle PCR was carried out as follows: denaturation at 92 °C for 30 s (1 min 30 s for the first cycle), annealing at 48 °C for 30 s and primer extension at 72 °C for 1 min 15 s. PCR products (5 µl) were analyzed by 1 % agarose gel electrophoresis, stained with ethidium bromide and visualized under a UV transilluminator.

**RFLP analysis:** PCR products 16r758f/M23Sr and FD9f3/r2 were digested with restriction enzymes. Aliquots of 5–20 µl, depending on the intensity of the DNA bands in agarose gels, were digested separately with 8 endonucleases: *Tru9I* (Promega), *TaqI*, *AluI* (Appligene), *RsaI*, *MaeI*, *HindIII* (Roche), *DraI* and *HpaII* (Gibco), according to manufacturers instructions. The restriction products were subsequently separated by 10 % polyacrylamide gel electrophoresis in TBE (Tris-Borate 90 mM, EDTA 1 mM) buffer, stained with ethidium bromide and visualized under a UV transilluminator.

**Cluster analysis:** The obtained RFLP patterns were utilized to perform a cluster analysis and to construct two different dendrograms, derived from each of the two DNA regions. The data were organized in square similarity matrices, using the algorithm:  $F = 2N_{n,n+1} / (N_n + N_{n+1})$  where  $F$  is the similarity coefficient of strains  $n$  and  $n+1$ ,  $N_{n,n+1}$  is the number of digestion bands shared by the two strains,  $N_n$  and  $N_{n+1}$  are the number of bands obtained from the digestion of all the 8 endonucleases in strains  $n$  and  $n+1$  respectively (LEE *et al.* 1998). The matrices were then processed with the program Systat 7.0.

**STOLD classification:** In order to assign STOLD to a definitive grouping, DNA extracted from infected periwinkle was amplified with the primers R16F2n (LEE *et al.* 1998) and R16R2 (LEE *et al.* 1993) and aliquots of the amplicon were separately digested with *Tru9I* and *AluI*. DNA from EY1, FD70, STOLC and AY1 infected periwinkles was used as references. The results were compared to the RFLP patterns described by LEE *et al.* (1998).

## Results

**PCR analysis:** Using primer pair P1/P7 followed by 16r758f/M23Sr, the expected product of about 1050 bp was obtained for all the samples, as shown in Fig. 1 A. Using

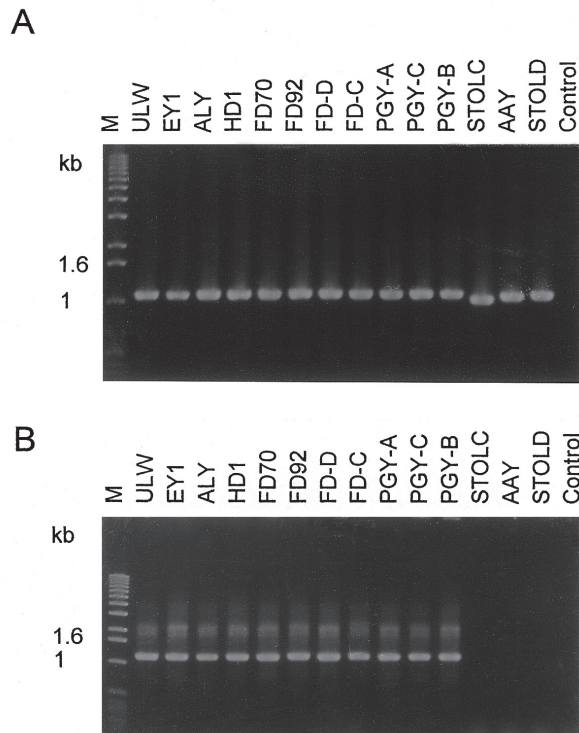


Fig. 1: Agarose gels of final DNA products of nested-PCR in 14 phytoplasma isolates. **A:** rDNA amplicons (1050 bp) obtained with primers 16S758f/M23Sr. **B:** DNA amplicons (1150 bp) obtained with primers FD9f3/r2. M: 1 kb ladder (BRL). Phytoplasmas: European elm yellows, ULW; American elm yellows, EY1; Italian alder yellows, ALY; hemp dogbane yellows, HD1; Flavescence dorée: FD70, FD92 (France), FD-D, FD-C (Italy); Palatinate grapevine yellows, PGY-A, PGY-C, PGY-B; tomato stolbur from France, STOLC; American aster yellows, AAY; tomato “stolbur” from Brazil, STOLD. Control: sterilized water instead of DNA in PCR mix.

primer pair FD9f2/r followed by FD9f3/r2, a band of about 1150 bp was obtained from all the EY-group isolates; DNA from AAY, STOLC and STOLD phytoplasmas was not amplified (Fig. 1 B).

**RFLP analysis:** The restriction profiles of the 16r758f/M23Sr DNA fragment in the ribosomal region are summarized in Tab. 2 and patterns obtained with *Tru9I* and *TaqI* are shown in Fig. 2 A and B, respectively. None of the isolates examined showed any restriction site for *DraI*, while *HindIII* cut only the STOLC and STOLD fragments. Altogether, a low variability was observed; nevertheless, EY-group phytoplasmas were clearly separated from phytoplasmas of other groups with the enzymes *Tru9I* (Fig. 2 A), *RsaI*, *HpaII* and *AluI*. Among EY-group phytoplasmas, *TaqI* (Fig. 2 B) and *MaeI* separated elm phytoplasmas (EY1, ULW) from non-elm phytoplasmas (ALY, HD1, FD70, FD92, FD-D, FD-C, PGY-A, PGY-C, PGY-B). In addition, within the latter cluster, *TaqI* separated FD-92 and FD-D, which showed a common restriction pattern, from other isolates. Among non-EY-group phytoplasmas, STOLD gave a pattern similar to that of elm phytoplasma when digested with *TaqI* and STOLC a pattern similar to that of elm phytoplasma when digested with *MaeI*.

The RFLP profiles of the DNA fragment amplified in the non-ribosomal region of EY-group phytoplasmas with primer

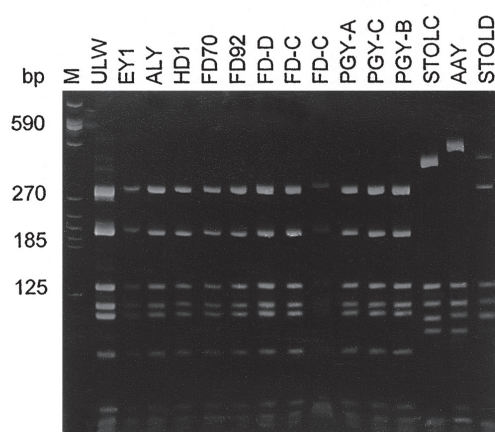


Table 2

Arbitrary designations for groups of restriction fragment length polymorphism of 16S758f/M23Sr amplimers obtained from 14 phytoplasma isolates after digestion with 8 restriction enzymes. Each number represents a distinct profile obtained with each restriction enzyme. Phytoplasmas, for abbreviations see Fig. 1

Phytoplasma	Restriction enzyme							
	<i>Dra</i> I	<i>Hind</i> III	<i>Tru</i> 9I	<i>Rsa</i> I	<i>Hpa</i> II	<i>Alu</i> I	<i>Mae</i> I	<i>Taq</i> I
ULW	I	I	I	I	I	I	I	I
EY1	I	I	I	I	I	I	I	I
ALY	I	I	I	I	I	I	II	II
HD1	I	I	I	I	I	I	II	II
FD70	I	I	I	I	I	I	II	II
FD92	I	I	I	I	I	I	II	III
FD-D	I	I	I	I	I	I	II	III
FD-C	I	I	I	I	I	I	II	II
PGY-A	I	I	I	I	I	I	II	II
PGY-C	I	I	I	I	I	I	II	II
PGY-B	I	I	I	I	I	I	II	II
STOLC	I	II	II	II	II	II	I	IV
AAY	I	I	III	III	III	III	III	V
STOLD	I	II	IV	IV	IV	IV	IV	I

A



B

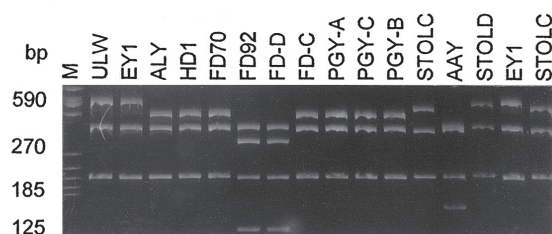


Fig. 2: Polyacrylamide gel (10 %) showing the RFLP patterns of phytoplasma rDNA amplimers shown in Fig. 1 A. **A:** after digestion with *Tru*9I. **B:** after digestion with *Taq*I. M: pBR322/*Hae*III (Appligene). Phytoplasmas, for abbreviations see Fig. 1.

pair FD9f3/r2 are summarized in Tab. 3 and patterns obtained with *Tru*9I and *Taq*I are shown in Fig. 3 A and B, respectively. While the enzymes *Hind*III and *Hpa*II did not show

any restriction site, all other digestions produced several distinct pattern types. In particular, *Tru*9I produced different profiles for all the isolates (9 out of 11), except for FD-D, FD92 and ALY, which were similar (Fig. 3 A). Several different patterns were also delineated with *Alu*I, *Mae*I, *Dra*I, *Taq*I (Fig. 3 B) and *Rsa*I. Elm phytoplasmas (EY1 and ULW) almost always showed a similar restriction pattern that was different from all others and they could be distinguished from each other only by means of *Tru*9I (Fig. 3 A). It must be emphasized that only FD92 and FD-D showed the same patterns with all the restriction enzymes used. The two French FD isolates (FD70 and FD92) were different only if digested with *Tru*9I and *Alu*I. FD70 showed almost always a digestion pattern similar to that of the Italian alder phytoplasma ALY, except with *Tru*9I. The two Italian FD isolates (FD-C and FD-D) revealed different patterns with 4 endonucleases out of 8; this was also the case between FD70 and FD-C. All the FD *sensu stricto* phytoplasmas, together with ALY, showed similar profiles when digested with *Dra*I and *Mae*I. The three grapevine phytoplasmas from Palatinate most often showed either two or three quite different profiles, clearly distinguishable also from the patterns obtained with the other isolates: PGY-A, PGY-B and PGY-C restrictions were different from the 4 FD isolates for 4 enzymes, 5 enzymes and 5 enzymes, respectively. The profile of hemp dogbane phytoplasma HD1 was unique with *Tru*9I, *Dra*I and *Mae*I.

**Cluster analyses:** The dendrogram obtained from RFLP data in the ribosomal region 16r758f/M23Sr (Fig. 4) revealed only some main groups. The three German grapevine phytoplasmas, the French FD70, the Italian FD-C, the Italian alder yellows ALY and the American hemp dogbane HD1 were grouped together, close to the French FD92 and the Italian FD-D, which anyway formed together a distinct cluster. The two elm phytoplasmas (EY1 and ULW) clustered together in a superior branch. The highest difference

Table 3

Arbitrary designations for groups of restriction fragment length polymorphism of FD9f3/r2 amplimers obtained from 11 phytoplasma isolates in group EY (16S rV) after digestion with 8 restriction enzymes. Each number represents a distinct profile obtained with each restriction enzyme. Phytoplasmas, for abbreviations see Fig. 1

Phytoplasma	Restriction enzyme							
	<i>Hind</i> III	<i>Hpa</i> II	<i>Rsa</i> I	<i>Taq</i> I	<i>Mae</i> I	<i>Dra</i> I	<i>Alu</i> I	<i>Tru</i> 9I
ULW	I	I	I	I	I	I	I	I
EY1	I	I	I	I	I	I	I	II
ALY	I	I	II	II	II	II	II	III
HD1	I	I	I	I	III	III	II	IV
FD70	I	I	II	II	II	II	II	V
FD92	I	I	II	II	II	II	III	III
FD-D	I	I	II	II	II	II	III	III
FD-C	I	I	I	III	II	II	IV	VI
PGY-A	I	I	III	II	IV	IV	IV	VII
PGY-C	I	I	I	IV	V	V	V	VIII
PGY-B	I	I	I	I	VI	VI	VI	IX

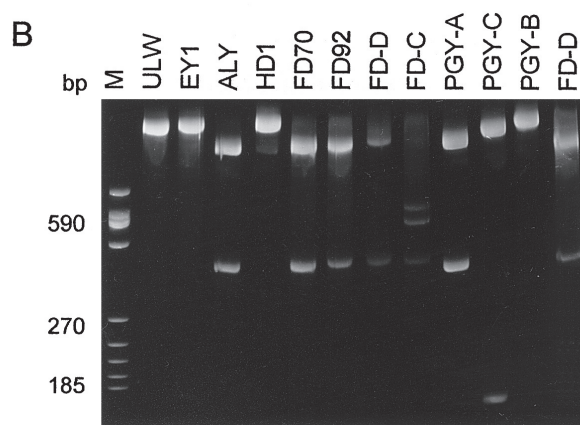
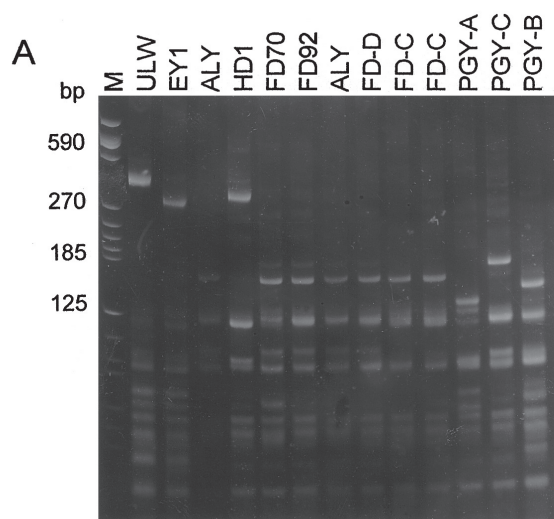


Fig. 3: Polyacrylamide gel (10 %) showing the RFLP patterns of non-ribosomal DNA FD9 amplimers of EY-group phytoplasmas shown in Fig. 1 B. **A**: after digestion with *Tru*9I; **B**: after digestion with *Taq*I. M: pBR322/*Hae*III (Appligene). Phytoplasmas, for abbreviations see Fig. 1.

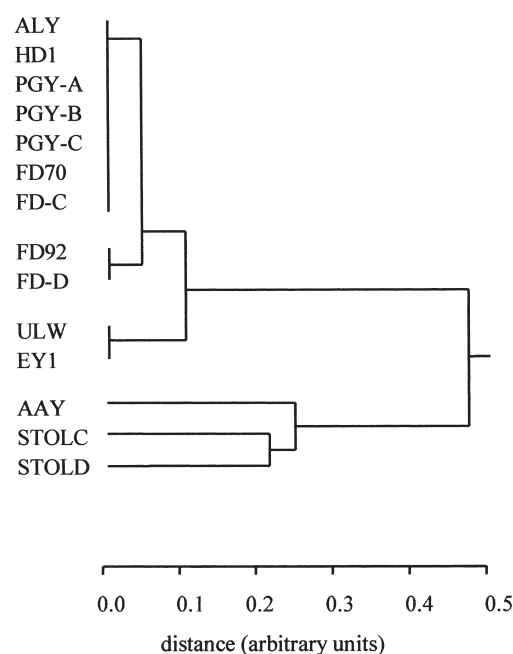


Fig. 4: Dendrogram obtained from cluster analyses of RFLP data of 16S758f/M23Sr rDNA fragment in 14 phytoplasmas shown in Tab. 1. Phytoplasmas, for abbreviations see Fig. 1.

occurred between phytoplasmas belonging to the 16SrV group on one side and AAY, STOLD and STOLC on the other side, which also were clearly different from each other.

The dendrogram obtained by RFLP data in the non-ribosomal region FD9f3/r2 (Fig. 5) showed minor differences among isolates. The results clearly revealed three distinct main clusters. The first cluster included the 4 *FD sensu stricto* and the Italian alder yellows ALY; FD92 and FD-D were in the same branch, quite close to FD70 and ALY, while FD-C was more different. The second cluster was less homogeneous and included the three German grapevine phytoplasmas PGY-A, PGY-B and PGY-C and the American hemp dogbane phytoplasma HD1. The two American and European elm

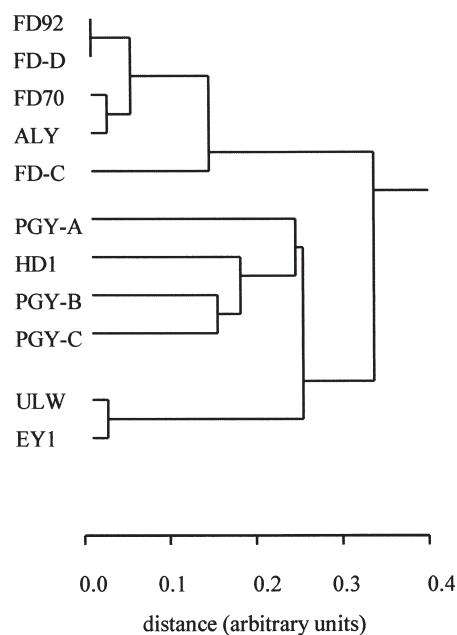


Fig. 5: Dendrogram obtained from cluster analyses of RFLP data of FD9f3/r2 non-ribosomal DNA fragment in 11 EY-group phytoplasmas shown in Table 2. Phytoplasmas, for abbreviations see Fig. 1.

phytoplasmas EY1 and ULW, very close to each other, formed the last cluster.

**STOLD classification:** None of the reference phytoplasmas used was shown to share the same RFLP pattern as STOLD with the endonucleases utilized. RFLP profiles with *Tru9I* and *AhaI* of the R16F2n/R16R2 rDNA fragment were compared with the profiles published by LEE *et al.* (1998). The results (not shown) classified STOLD into the ribosomal group 16SrVI-A, whose representative is the clover proliferation phytoplasma (CP, Canada).

### Discussion

The occurrence of different FD isolates in France has first been shown with serological tools (BOUDON-PADIEU *et al.* 1987; 1989; 1990; Osler *et al.* 1992), then with southern blot and PCR-RFLP analyses (DAIRE *et al.* 1992, 1997 b; DAIRE 1994). Isolates FD70, FD88 and FD92 (similar to FD88) have been obtained over a 20-year period, from wild *S. tatanus* specimen trapped in FD-affected vineyards. In Italy, two FD isolates (FD-C and FD-D) have been detected in diseased vines (DAIRE *et al.* 1997 b, MARTINI *et al.* 1998), both transmitted by *S. tatanus* (MORI *et al.* 2000). Several comparative studies have investigated the diversity of grapevine and non-grapevine EY phytoplasmas (DAIRE *et al.* 1997 b, MARCONE *et al.* 1997 a; GRIFFITHS *et al.* 1999; MARTINI *et al.* 1999). In particular, MAIXNER and REINERT (1999), REINERT (1999) and MAIXNER *et al.* (2000) have shown that German grapevine phytoplasmas PGY could originate from alder phytoplasmas in Germany. The present report is the first study carried out to compare all the EY-group grapevine isolates reported until now and European or American non-grapevine isolates.

Several EY-group phytoplasmas were included as non-grapevine isolates, among which was STOLD (BOUDON-PADIEU *et al.* 1996). As RFLP analyses of STOLD 16r758f/M23Sr fragment were not in agreement with classification into the EY group, the R16F2n/R16R2 rDNA fragment was analysed and STOLD eventually classified into the 16SrVI-A group according to the data of LEE *et al.* (1998). This comparative study addressed two different regions in phytoplasma DNA. The 16S-23S spacer region was chosen because it is variable as compared to the coding region of the 16S rRNA, especially in the case of EY-group phytoplasmas as shown by SEEMÜLLER *et al.* (1998). A second promising DNA fragment was the variable non-ribosomal FD9 region (DAIRE *et al.* 1997 b). However, direct PCR with the original FD9f/r primers, happened to lack sensitivity for detection in a number of FD-affected field-grown vines. In order to develop a more sensitive procedure, one new primer on the forward strand and two new primers on the reverse strand of the FD9 fragment were constructed, using the aligned sequences on 5 isolates, obtained by REINERT (1999). The 5 primers shown in Tab. 1, were assayed in combinations for several direct and nested-PCR procedures (unpubl.) and the two pairs FD9f2/r and FD9f3/r2 were selected for use in nested-PCR. Their sensitivity in routine assays has been shown in a separate study (CLAIR *et al.* 2000).

In order to reveal the higher number of differences, a panel of 8 restriction enzymes was chosen on the basis of previous studies on ribosomal DNA and on FD9 DNA fragment of EY phytoplasmas (LEE *et al.* 1998; GRIFFITHS *et al.* 1999; DAIRE 1997 b and unpubl.). RFLP results were consistent with previous data comparing EY1, ULW, FD70, ALY and HD1 (MARCONE *et al.* 1997 a, b; GRIFFITHS *et al.* 1999; MAIXNER and REINERT 1999). Restriction patterns obtained with the FD9f3/r2 fragment were consistent with data obtained by DAIRE *et al.* (1997 b) on the longer FD9f/r fragment. As a whole, RFLP results showed that FD70 and FD-C were different, while FD92 and FD-D were similar. The similarity between FD-D and FD88 (=FD92) has been suggested formerly on the basis of PCR/RFLP analyses of the same FD9f/r region by MARTINI *et al.* (1999). The difference between FD-C and FD-70 was recently confirmed by further results obtained by MARTINI (pers. comm.).

Cluster analyses of data on the FD9 fragment indicated the existence of three main subgroups. Within the first subgroup, the 4 FD isolates clustered together with ALY; FD70 seemed to be very close to FD-D and FD-92, while a higher difference was observed with FD-C. The utility of using other DNA regions but the ribosomal one for such analyses is quite clear. However, it is interesting to notice that, on the basis of the data obtained in the 16S-23S region, FD92 and FD-D clustered separately from all other grapevine isolates because of the presence of a *TaqI* restriction site, while PGY and HD1 were classified in the same group as FD70 and FD-C. We may wonder about the actual importance of a single restriction site, even if present in a quite conserved region like 16S rDNA. Moreover, it might be assumed that FD9 is a coding region. The first part of this fragment has a strict identity (55 %) with the DNA region coding for the 50S ribosomal protein L15 of *Mycoplasma capricolum*, and the



remaining part of FD9 fragment also shares some homology with the *Sec-Y* gene of translocase protein of the same *Mycoplasma* species (REINERT 1999). Hence, the classification based on that fragment could have enough biological and ecological meaning. Thus we assume that the dendrogram derived from the FD9 fragment RFLP data reflects in a better way the real differences among all phytoplasma isolates.

With regard to the relationships between FD *sensu stricto* on one side and PGY on the other, the cluster analysis based on FD9 restriction patterns showed that all the FD isolates clustered together, while the three PGY isolates were grouped in a separate branch. However, REINERT (1999) and REINERT and MAIXNER (2000) have obtained different results in a pair-wise comparison and a multiple comparison among the FD9 sequences of the three German PGY with FD70 and EY1. Obviously, DNA sequence should reflect differences more accurately than restriction patterns. However, the authors have reported conflicting results between their two sets of data. Moreover, the dendrogram shown in Fig. 5 matched exactly with other results obtained in our laboratory with a different approach (unpubl.).

The results confirmed that there is a common FD isolate in France and Italy, *i.e.*, FD92 = FD-D. This isolate, also detected in Spain (DAIRE *et al.* 1997 b), was the only grapevine EY-group phytoplasma identified in French vineyards in the last decade. Therefore, FD70 might be considered as a mere laboratory isolate; nonetheless, it appeared to be very closely related to FD92. Anyhow, though it has no apparent epidemiological significance at the moment, it might indeed be useful to understand phytoplasma evolution.

Another interesting part of these analyses is the strong similarity between FD isolates and ALY. ALY has been isolated from alder in South Italy (MARCONE *et al.* 1997 b) and no data are available on EY phytoplasma in grapevine in the same region. However, this phytoplasma could be transmitted to grapevine by alder leafhoppers, similarly to transmission of PGY by *O. alni* in Germany (MAIXNER *et al.* 2000). Because of the close relationships between ALY and FD, the presence of ALY phytoplasma in alder trees in regions infested with *S. titanus* could represent a threat for a potential outburst of FD. However, transmissibility of ALY by *S. titanus* remains to be demonstrated.

In conclusion, three different European FD *sensu stricto* phytoplasmas could be identified. Among these, only two have an epidemiological importance at the moment. One isolate (FD92 = FD-D) seems to be more common, being diffused in both France and Italy but also in Spain; the other isolate (FD-C) seems to be present only in some Italian regions. ALY, an Italian alder phytoplasma, was shown to be closer to the FD92 isolate than was FD-C. FD70, at the moment a "laboratory isolate", was also shown to be very close to FD92. Moreover, it was shown that the three PGY phytoplasmas, similar to phytoplasmas inhabiting symptomless alder in Germany, are distantly related both to FD *sensu stricto* and to ALY, an alder phytoplasma in Italy.

Finally, we must stress that it might be possible that there is some genetic polymorphism of phytoplasma in grapevines grown in the field. Though we used isolates that were thought to be representative of the field situation, a larger

screening of field samples, already started in our laboratories, might reveal other interesting phytoplasma isolates which would be useful in understanding the evolution of this group and some critical aspects of the epidemiology of FD disease, such as long distance transportation and local diffusion.

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