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Detection and differentiation of grapevine yellows phytoplasmas belonging to the elm yellows group and to the stolbur subgroup by PCR amplification of non-ribosomal DNA

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Abstract

Primer pairs were designed from a cloned DNA probe of a strain of flavescence dorée (FD) phytoplasma and from a cloned DNA probe of a strain of stolbur phytoplasma. Among an array of reference phytoplasma strains maintained in periwinkle, pair FD9f/r amplified a 1.3 kb DNA fragment only with phytoplasma strains of elm yellows (EY) group, i. e. two strains of FD and two strains of EY. *Tru*9I restriction analysis of the fragment amplified by FD9f/r revealed a diversity among EY-group phytoplasmas. The FD strains differed from the strains isolated from elm. The profile of the phytoplasmas infecting the grapevine samples from Catalonia and most of the samples from Northern Italy were identical to that of a FD strain. Three other profiles were detected in grapevine from Palatinate, in Germany.

The two primer pairs derived from a stolbur strain, STOL4f/r and STOL11f2/r1, specifically amplified a 1.7 kb and a 0.9 kb DNA fragment, respectively, with all strains in the stolbur subgroup. However, the pair STOL4f/r did not recognise strain MOL. Both pairs allowed to detect phytoplasmas in diseased grapevines from France, Italy, Spain and Israel. Attempts to differentiate between phytoplasmas in the stolbur subgroup by restriction analyses failed. The pairs FD9f/r and STOL11f2/r1 could be used in the same reaction (multiplex PCR) to detect EY-group phytoplasmas, stolbur-subgroup phytoplasmas or both phytoplasmas simultaneously when template DNAs were mixed.

Introduction

Grapevine yellows (GY) are diseases of *Vitis vinifera* L. which show similar symptoms and are caused or supposed to be caused by non-cultivated phytoplasmas (Caudwell et al., 1971). Flavescence dorée (FD) was the first GY described, in France, in the late fifthties (Caudwell, 1957) and it was shown to be transmitted by the leafhopper *Scaphoideus titanus* Ball. A few years later, two other GY, the bois noir disease (BN) in Northern France (Caudwell, 1961) and Vergilbungskrankheit in Germany (Gärtel, 1965) were reported and could be distinguished from FD because transmission attempts by *S. titanus* failed. GY have since been reported in most of the viticultural areas.

Only recently analyses of amplified 16S rDNA allowed to show that different phytoplasmas are associated with GY (Daire et al., 1993a and 1993b; Prince et al., 1993). It appeared that in Europe most of the detected phytoplasmas could be assigned either to the elm yellows (EY) phytoplasma group or to a particular subgroup of the aster yellows phytoplasma group, that is the stolbur phytoplasma subgroup (Seemüller et al., 1994). The latter is also named 16S rRNA group I-g by other authors (Bertaccini et al., 1993) following the classification system of Lee et al. (1993). To

the EY group belong the FD phytoplasma (Seemüller et al., 1994) and also GY phytoplasmas occurring in different regions of Italy (Daire et al., 1993b; Bianco et al., 1993; Bertaccini et al., 1995; Daire et al., 1997) and in the Palatinate area in Germany (Maixner et al., 1995a) whereas the agents of bois noir disease and Vergilbungskrankheit were found to belong to the stolbur subgroup along with phytoplasmas detected in various regions of France (Daire et al., 1993a; 1997) and of Italy (Daire et al., 1993b; Bianco et al., 1993; Bertaccini et al., 1995; Daire et al., 1997), in Catalonia (Laviña et al., 1995) and in Israel (Daire et al., 1993b; 1997). Thus rDNA primers have shown to be most valuable for detection and classification of grapevine phytoplasmas. However since this region is highly conserved, it may be not suited for differentiating phytoplasma strains within a given group, which would be of great interest for epidemiological studies. In the present paper we report of the development of PCR primer pairs which derived from randomly cloned DNA probes previously isolated from a FD strain and a stolbur strain. Their usefulness for the detection and the differentiation of GY phytoplasmas was assessed on grapevine samples from diverse European regions and from Israel.

Materials and methods

Grapevine samples

Leaf samples of various cultivars of *V. vinifera* showing symptoms of GY were collected in different viticultural regions of France, in the german Palatinate, in the spanish Catalonia province, in Northern Italy and in Golan vineyards in Israel. Certified grapevine material and grapevine without symptoms were used as healthy control.

Reference phytoplasma strains in periwinkle

Different phytoplasma strains, previously transmitted to periwinkle (*Catharanthus roseus* L.) and assigned to different phytoplasma groups in the phylogenetic classification by Seemüller et al. (1994) were included in the study. AAY, American aster yellows (Florida) and EAY, European aster yellows (Germany) from aster yellows group. CH1, grapevine yellows from Emilia-Romagna (Italy); IPVR, Italian periwinkle virescence; MOL, Molières disease of cherry (France); STOL, stolbur of pepper (Croatia) and STOLF, stolbur of tomato (France), all from stolbur subgroup (= 16S rRNA group I-g) of aster yellows group. FD70 and FD88, two strains of grapevine flavescence dorée (both from France); EY, elm yellows (New York) and ULW, elm yellows (France), all from the elm yellows group. ASHY, ash yellows (New York) from the ash yellows group. PYLR, peach yellow leaf roll (California) and GYU (= FDU), grapevine yellows from Udine (Italy), both from the western X-disease group. AP, apple proliferation (Germany) from the apple proliferation group.

Strains FD70 and FD88 were described by Daire et al.(1992a). IPVR was described by Davis et al. (1992) and CH1 by Prince et al. (1993). Further information on all other strains can be found in Schneider et al.(1993). A periwinkle was doubly infected with FD and stolbur (STOL strain) by grafting with two shoots taken on a FD-infected periwinkle and on a STOL-infected periwinkle. Tissues of periwinkle seedlings were used as healthy controls.

DNA extraction

Approximately 1.5 g of shoots, leaves and apices from periwinkle were ground, using mortar and pestle, in 6 ml of extraction buffer (2% cethyltrimethylammonium bromide, 100 mM Tris-HCl, pH 8, 10 mM EDTA, pH 8, 1.4 M NaCl, 0.1% 2-mercaptoethanol) preheated at 65 °C. This brei was transferred into a centrifuge tube and incubated for 10 min at 65 °C. After an extraction with an equal volume of chloroforme, the aqueous phase was recovered and the nucleic acids precipitated with an equal volume of isopropanol and collected by centrifugation. The pellet was washed with 70% ethanol, dried and dissolved in deionized water. Grapevine DNA was extracted from a phytoplasma-enriched fraction of leaf main veins, according to Daire et al. (1992a).

Construction of primers and PCR specificity

The primers described herein derived from strain FD70 or strain STOL DNA fragments which had been randomly cloned in pUC18 and used as hybridisation probes (Daire, 1994). These cloned DNA fragments were partially sequenced from both ends using the fmol DNA sequencing system from Promega. Primer pair FD9f/r derived from the cloned *Eco*RI DNA fragment FD9 (1.3 kb). The pair STOL4f/r derived from the *Hind*III DNA fragment STOL4 (1.7 kb) and the pair STOL11f2/r1 from the *Hind*III DNA fragment

Table 1. Sequences of the primers

Primer	Primer sequence (5′–3′)
FD9f	GAATTAGAACTGTTTGAAGACG
FD9r	TTTGCTTTCATATCTTGTATCG
STOL4f	TTTAGCGATATTGGGAGAA
STOL4r	ATCCTTGAATTCTTTGACG
STOL11f2	TATTTTCCTAAAATTGATTGGC
STOL11r1	TGTTTTTGCACCGTTAAAGC

STOL11 (0.9 kb). All the primers were close to fragment ends, so that the expected size of the amplified products was approximately that of the cloned fragments. Primer pair fU5/rU3 (Lorenz et al., 1995), which amplifies a ribosomal DNA fragment in all phytoplasma strains, was used as a control for the presence of phytoplasmas in the infected periwinkle plants. The sequences of the FD and STOL primers are given in Table 1.

PCR conditions

The reaction mixture contained 10–50 ng of template DNA, 0.25 μM of each primers, 250 μM of each dNTPs, 3.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 9), 50 mM KCl, 0.1% Triton X100, 0.2 g/ml BSA and 0.5 unit of *Taq* polymerase (Appligene, France). The reactions were performed in a total volume of 40 μl, overlaid with mineral oil. In all experiments a predenaturation step at 92 °C for 90 s was performed. Thirty cycles for detection in periwinkle and 40 cycles for detection in grapevine were then realised as follows: denaturation for 30 s at 92 °C, annealing for 30 s at 55 °C (for STOL11f2/r1) or 48 °C (for STOL4f/r) or 54 °C (for FD9f/r) and elongation for 80 s at 72 °C.

Conditions for multiplex PCR

STOL11f2/r1 and FD9f/r were used simultaneously. The conditions were as above, with an annealing temperature of 55 °C.

Analysis of PCR products

PCR products (7 μl) were analysed by electrophoresis in 1.5% agarose gel. For restriction analyses, the amplified DNA was directly digested by mixing 10 μl of PCR mixture with 1 unit of enzyme. A panel of 10 enzymes

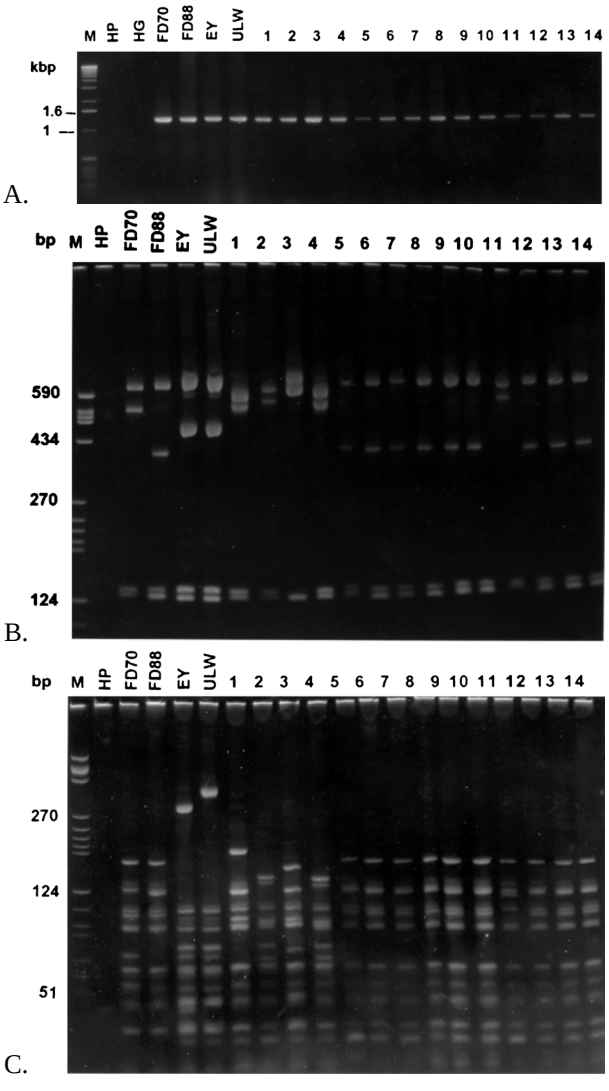

Figure 1. PCR product (1.3 kb) obtained with primer pair FD9f/r from phytoplasmas of the elm yellows group in periwinkle and in naturally infected grapevines of different cultivars. 1A: native amplication products. 1B: *Alu*I restriction profile of the products. 1C: *Tru9*I restriction profile of the same products.
M, molecular weight marker: 1kb ladder (BRL) in 1A and pBR322/*Hae*III (Appligene) in 1B and 1C. HP, healthy periwinkle. FD70 and FD88, flavescence dorée, strain FD70 and FD88 respectively; EY and ULW, elm yellows, strain EY and ULW respectively. All these strains were maintained in periwinkle. HG, healthy grapevine; 1, 2, 3 and 4, four different plants of cv Scheurebe from Palatinate. 5, cv Grenache from Catalonia. 6, 7 and 8, three plants of cv Carignan from Catalonia. 9 and 10, two plants of cv Alicante bouschet from Pyrénées orientales, Southern France. 11, cv Perera from Udine, Italy. 12, cv Garganega from Fruili-Venezia Giula, Italy. 13, cv Trebiano and 14, cv San Giovese, both from Vicenza, Italy.

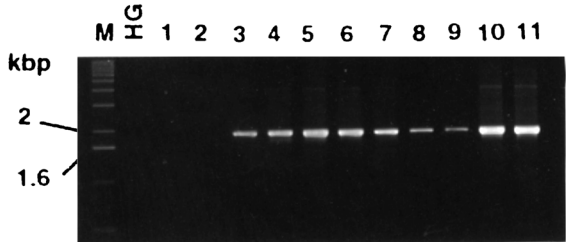

Figure 2. Detection of phytoplasmas in GY-diseased grapevines by amplification of a 1.7 kb fragment in PCR with primers STOL4f/r. M, molecular weight marker (1 kb ladder BRL). HG, healthy grapevine cv Chardonnay. 1 and 2 DNA from FD-positive grapevines: 1, cv Alicante bouschet from Pyrénées Orientales, Southern France; 2. cv Garganega from Fruili-Venezia Giula, Italy. 3, cv Gamay from Savoie, France. 4, cv Chardonnay from Côte d′or, France. 5, cv San Giovese from Emilia-Romagna, Italy. 6, cv Chardonnay from Fruili-Venezia Giula, Italy. 7, cv Chardonnay from Sicily. 8 and 9, two plants of cv Chardonnay from Catalonia. 10 and 11, two plants of cv Chardonnay from Israel. Samples in lanes 1 and 2 were used in Figure 1, lanes 9 and 12 respectively.

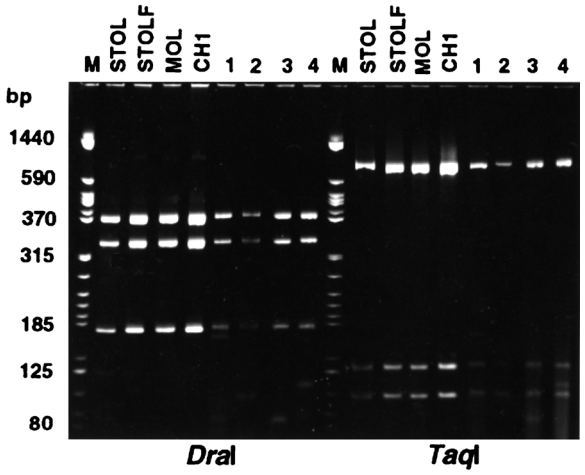

Figure 3. *Dra*I and *Taq*I restriction analyses of the PCR product (0.9 kb) amplified with primer pair STOL11f2/r1 from periwinkle and grapevines infected with a stolbur phytoplasma. M, molecular weight marker (pBR322/*Hae*III plus pBR322/*Taq*I, Appligene). STOL, stolbur. STOLF, stolbur. MOL, Molières disease. CH1, grapevine yellows. All these strains were maintained in periwinkle. 1, GY-diseased grapevine from France. 2, from Italy. 3, from Catalonia. 4, from Israel.

was used, among which enzymes known for their AT-rich restriction sites were included. *Alu*I, *Dra*I, *Eco*RI, *Eco*RV, *Hae*III, *Hha*I, *Kpn*I, *Mbo*I, *Taq*I (Appligene) and *Tru*9I (= *Mse*I) (Promega) were employed. After overnight incubation at 37 °C (or 65 °C for *Tru*9I and *Taq*I), the digested DNA was analysed by electrophoresis in 10% acrylamide gel, stained with ethidium bromide and visualised under U. V. light.

Results

Detection and differentiation of FD and related phytoplasmas

The primer pair FD9f/r was first checked with the reference strains maintained in periwinkle. As shown in Table 2, this pair allowed the amplification of a 1.3 kb fragment from both FD strains and also from the elm yellows strains, but not with the other strains examined or the healthy control. It was also possible to amplify a 1.3 kb fragment from an array of samples taken on naturally infected grapevines, collected in Catalonia, Southern France, Northern Italy and the Palatinate area in Germany (Figure 1A). Among the restriction enzymes tested, *Alu*I, and especially *Tru*9I appeared as the best suited enzymes for RFLP analysis of the fragment. Following *Alu*I restriction analysis (Figure 1B) six profiles could be delineated corresponding to i) strain FD70, ii) strain FD88 and the grapevines from Catalonia (lanes 5,6,7,8), from Southern France (lanes 9 and 10) and three grapevines from Northern Italy (lanes 12, 13 and 14), iii) strains EY and ULW, iv) two grapevines from Palatinate (lanes 1 and 4), v) one grapevine from Palatinate and one from Northern Italy (lanes 2 and 11 respectively) and vi) one grapevine from Palatinate (lane 3). *Tru*9I restriction analysis yielded more information since it allowed to delineate eight profiles with the same samples as above (Figure 1C), corresponding to i) FD70, ii) FD88 and the same samples as in the case of *Alu*I (lanes 5, 6, 7, 8, 9, 10, 12, 13 and 14), iii) strain EY, iv) strain ULW, v) one grapevine from Italy (lane 11); the three other profiles were found in three samples from Palatinate, that is vi) lane 1, vii) lane 2 and 4, and viii) lane 3.

Detection of stolbur and related phytoplasmas

The two primer pairs STOL4f/r and STOL11f2/r1 were checked with reference strains belonging to the stolbur subgroup and with reference strains in other groups (Table 2). STOL11f2/r1 allowed amplification of a 0.9 kb fragment from all members of the stolbur subgroup, that is strains STOL, STOLF, MOL, CH1, IPVR, and not with the other strains in periwinkle and the healthy control (Table 2). STOL4f/r allowed amplification of a 1.7 kb fragment in the same strains as above, except

Table 2. Detection of reference strains using FD and STOL primers

Strains	Group or subgroup	Primer pairs			
		fU5/rU3[a]	FD9f/r (1.3[b])	STOL4f/r (1.7)	STOL11f2/r1 (0.9)
HP		-	-	-	-
CH1	Stolbur	+	-	+	+
IPVR	Stolbur	+	-	+	+
MOL	Stolbur	+	-	-	+
STOL	Stolbur	+	-	+	+
STOLF	Stolbur	+	-	+	+
AAY	Aster yellows	+	-	-	-
EAY	Aster yellows	+	-	-	-
FD70	Elm yellows	+	+	-	-
FD88	Elm yellows	+	+	-	-
EY	Elm yellows	+	+	-	-
ULW	Elm yellows	+	+	-	-
ASHY	Ash yellows	+	-	-	-
PYLR	Western X	+	-	-	-
GYU	Western X	+	-	-	-
AP	Apple proliferation	+	-	-	-

[a] phytoplasma specific 16S rDNA pair fU5/rU3.
[b] length in kb of the amplified fragment.
+/- presence / absence of amplification product.

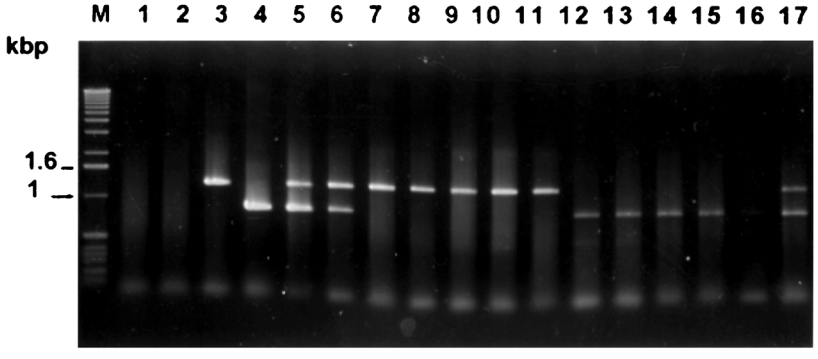

Figure 4. Detection of a FD phytoplasma and of a stolbur phytoplasma by multiplex amplification combining pairs FD9f/r and STOL11f2/r1. M, molecular weight marker (1 kb ladder BRL). 1, healthy periwinkle DNA. 2, healthy grapevine DNA. 3, FD70-infected periwinkle DNA. 4, STOL-infected periwinkle DNA. 5,6, two different templates of DNA prepared from a FD70-plus-STOL double-grafted periwinkle. 7 to 11, DNA from grapevine which tested for a EY-group phytoplasma in other experiments: 7, cv Cinseau from Aude, Southern France; 8 and 9 , two plants of cv Carignan from Catalonia; 10 and 11, two plants of cv Alicante bouschet from Aude, Southern France. 12 to 16, DNA from grapevine which tested for a stolbur-subgroup phytoplasma in other experiments: 12 and 13, two plants of cv Cabernet Sauvignon from Loire region, 14 and 15, two plants of cv Chardonnay from Bourgogne, France. 16, cv Chardonnay from Ardèche, France. 17, mixed DNA templates from samples in lane 11 and lane 14.

for strain MOL which appeared in that respect distinct from the other strains in the stolbur subgroup.

In grapevine, STOL4f/r amplified a 1.7 kb fragment with DNA of diseased samples originating from several regions of France and Italy, and from Spain and Israel (Figure 2). A 0.9 kb fragment was successfully amplified using STOL11f2/r1 and the grapevine samples found positive with STOL4f/r (not shown).

Attempts to differentiate between reference strains and grapevine isolates in the stolbur subgroup were done by restriction analyses of the fragments amplified with STOL4f/r and STOL11f2/r1 from strains STOL, STOLF, MOL (with STOL11f2/r1 only), CH1 and four grapevine samples collected in France, Italy, Spain and Israel respectively. All the enzymes cited above were used. None of these analyses could demonstrate any

RFLP among these phytoplasma DNA fragments. An example of the uniform results is shown in the case of STOL11f2/r1 amplification products restricted with *Dra*I and *Taq*I (Figure 3). The lower bands detectable in lanes 1 to 4 in *Dra*I profiles were considered to be artifacts.

Attempts of detection of a stolbur subgroup phytoplasma with stol11f2/r1 were made on a number of grapevine samples which had tested positive with FD9f/r, among which all the samples presented in Figure 1. No positive response was obtained. Similarly, no positive amplification could be obtained with FD9f/r primers on a number of stolbur positive samples (not shown).

Multiplex PCR using primer pairs FD9f/r and STOL11f2/r1

Figure 4 shows the results obtained when both FD9f/r and STOL11f2/r1 were mixed for amplification. Detection of either a EY-group phytoplasma or a stolbur-subgroup phytoplasma was achieved with DNA of FD and STOL strains maintained in periwinkle and from GY-diseased grapevines. When the template DNAs from FD or STOL periwinkles or grapevines were mixed in the reaction, or when template DNA was extracted from the doubly infected periwinkle, a simultaneous amplification of both fragments was obtained. No amplification occurred in healthy periwinkle and grapevine.

Discussion

Previous Southern hybridisation data had demonstrated that strains FD70 and FD88 in periwinkle presented genetic dissimilarities and that these strains were also distinct, though closely related, from EY strains (Daire et al., 1992b). However, for further investigation of the variability occurring among grapevine phytoplasmas, owed to the low concentration of phytoplasmas in grapevine, hybridisation is often not sensitive enough for their detection and PCR turned out to be far more adequate. Several authors indeed reported sensitive detection of phytoplasmas belonging to the EY group in grapevine in France and Italy using 16S rDNA primers, either universal (Daire et al., 1993a and b; Bertaccini et al., 1995) or EY group specific (Bertaccini et al., 1995; Bianco et al., 1993). However, no differentiation between these phytoplasmas could be obtained, probably due to the relatively high degree of conservation of the regions studied. In contrast, we found that a pair of primers derived from a randomly cloned FD probe span a sequence in non ribosomal DNA more variable than rDNA and therefore afford to differentiate phytoplasmas within the EY group. The results demonstrate a relative diversity among the phytoplasmas within the EY group infecting grapevine and show that, so far, they are distinct from the strains isolated from diseased elms. This is consistent with the unsuccessful attempts to amplify DNA from a EY-group phytoplasma in grapevine in Italy using a non-ribosomal primer pair deriving from an American strain of EY (Bertaccini et al., 1995).Thus it appeared that the EY-group phytoplasmas found in grapevine in Southern France, in Catalonia and most of the samples from Northern Italy are similar or identical to one of the two FD strains (FD88) maintained in the periwinkle. A fact which strongly suggests that the FD disease first described in France, i.e. the GY transmitted by *S. titanus*, is present in Northern Italy and in Catalonia. FD-infected *S. titanus* trapped in vineyards have been detected in large numbers in France and in Italy with specific antibodies (Boudon-Padieu et al., 1989; Osler et al., 1992) or FD-specific probes and primers (Daire, 1994). *S. titanus* is also a very abundant species in Catalonia (Lavina et al., 1995) and is very likely the vector of this disease.

Maixner et al. (1995a), reported the first detection of a FD-type phytoplasma occurring in the Palatinate region of Germany. Analysing samples from the same area in the present study enabled to show that this phytoplasma differed from FD and EY reference strains. The dissimilarities between FD and this German phytoplasma suggest that it was not imported via infected stocks from France or Italy. As *S. titanus* is not present in Germany, another vector, yet unknown must be responsible for transmission.

The FD primers could be used to assess the relationships between EY-group phytoplasmas which occur in various plant species (Mäurer et al., 1993; Poggi Pollini et al., 1996) and to search for their vectors. It is now important to address the problem of the biological signification of the variability in the EY group. FD is the only EY-group disease for which transmission by its vector, the ampelophagous species *S.titanus*, is well understood. Transmission trials with different EY-group phytoplasmas and *S. titanus* specimens should be undertaken.

The stolbur primers proved to specifically react with members of the stolbur subgroup and their use confirmed the occurrence of such phytoplasmas in

GY-affected grapevines in European countries and Israel. The restriction analyses suggest that, in contrast with the EY group, the variability within the stolbur subgroup is rather low. The strain MOL isolated from cherry was the only strain found to be different from the others. It was not detected in the grapevine samples analysed in this study. The stolbur phytoplasma can cause Yellows in a wide range of wild and cultivated plant species (Fos et al., 1992) and a link between these diseases and BN occurrence is strongly suspected since the polyphagous planthopper *Hyalesthes obsoletus*, the main vector of the stolbur phytoplasma, was successfully used to inoculate such a phytoplasma to grapevine in Germany (Maixner et al., 1995b) and in France (Sforza et al., 1996).

In the present investigations and in a wide survey of GY in several viticultural countries (Daire et al., 1997) we found that phytoplasmas in the EY group and in the stolbur subgroup were the most frequently occurring in GY diseased grapevines. For this reason the multiplex PCR reaction described here could be useful for routine diagnosis since it allows detection of either EY group or stolbur subgroup phytoplasmas in a single reaction.

Several authors (Bianco et al., 1993; Bertaccini et al., 1995) have reported cases of double infection in grapevines, evidenced by means of nested PCR with ribosomal primers. So far we have not detected a second phytoplasma in a naturally infected grapevine. This suggests that if any sample we analysed were actually doubly infected, one of the phytoplasmas would be present at a too low titre for direct-PCR detection. Nested PCR is likely the tool of choice to address the problem of multiple infection. However one should be aware that extreme care must be taken to avoid false positive amplification with these methods and that the biological significance of a second phytoplasma at a very low titre must be evaluated.

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