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Real-time PCR detection systems for Flavescence dorée and Bois noir phytoplasmas in grapevine: comparison with conventional PCR detection and application in diagnostics

M. Hren[a]*, J. Boben[a], A. Rotter[a], P. Kralj[b], K. Gruden[a] and M. Ravnikar[a]

[a]*National Institute of Biology, Department of Plant Physiology and Biotechnology, Večna pot 111, 1000 Ljubljana; and* [b]*Jozef Stefan Institute, Department of Knowledge Technologies, Jamova 39, 1000 Ljubljana, Slovenia*

A new real-time PCR detection system was developed for grapevine yellows (GY) using TaqMan minor groove binder probes and including two amplicons for group-specific detection of Flavescence dorée (FD) and Bois noir (BN) phytoplasmas, plus a universal phytoplasma amplicon. FD and BN amplicons were designed to amplify species-specific genomic DNA fragments and the universal amplicon to amplify the 16S ribosomal DNA region. Efficiency of PCR amplification, limit of detection, range of linearity and dynamic range were assessed for all three amplicons. The specificity of detection systems was tested on several other isolates of phytoplasmas and bacteria and on healthy field grapevine and insect samples. No cross-reactivity with other phytoplasma strains, plant or insect DNA was detected. The assay was compared with conventional PCR on more than 150 field grapevine, insect and field bindweed samples. Real-time PCR showed higher sensitivity as phytoplasmas were detected in several PCR-negative and in all PCR-positive samples. A data-mining analysis of results from both detection approaches also favoured real-time PCR over conventional PCR diagnostics. The developed procedure for detection of phytoplasmas in grapevine also included amplification of plant DNA co-extracted with phytoplasmic DNA, providing additional quality control for the DNA extraction and PCR amplification for each sample. The newly developed assay is a reliable, specific and sensitive method easily applicable to high-throughput diagnosis of GY.

Keywords: data mining, diagnostics, grapevine yellows, real-time PCR, stolbur

Introduction

Grapevine yellows (GY) is a term for a group of diseases which are caused by different phytoplasmas but which show similar symptoms in infected grapevine plants. Flavescence dorée (FD) and Bois noir (BN) are the main causes of GY in Europe (Daire *et al*., 1997; Martini *et al*., 1999; Angelini *et al*., 2001; Boudon-Padieu, 2003; Lee *et al*., 2004).

Phytoplasmas are found in the phloem sieve tubes of plants (Lee *et al*., 2000; Garnier *et al*., 2001; Christensen *et al*., 2005) and in phloem-sucking homopterous insects (Webb *et al*., 1999; Christensen *et al*., 2005). Several distinct phytoplasma groups have been identified on the basis of RFLP or phylogenic analysis of 16S rRNA sequences (Lee *et al*., 1998; Seemüller *et al*., 1998). FD belongs to group 16SrV (elm yellows group) and BN to group 16SrXII-A (stolbur group). In 2004, the '*Candidatus* (*Ca*.) Phytoplasma' taxon was introduced at the genus level and several taxons were introduced at the species level, based on genetic (16S rRNA sequence similarity) and biological properties (e.g. diseases caused in plants). The Flavescence dorée phytoplasma was classified into taxon '*Ca*. Phytoplasma vitis' and Bois noir into '*Ca*. Phytoplasma solani' (IRPCM, 2004; Lee *et al*., 2004).

FD has the greatest economic impact among GY-causing phytoplasmas. The most important reasons are high yield loss and high epidemiological potential because of the ubiquitous presence of the cicadellid *Scaphoideus titanus*, its transmission vector in European vineyards. In addition, the application of insecticides to control the insect vector adds to the costs of production (Lee *et al*., 2000; Seemüller *et al*., 2002; Boudon-Padieu, 2003; EPPO/CABI, 2003). FD is recognized by the European and Mediterranean Plant Protection Organization (EPPO) as a quarantine pest (EPPO/CABI, 2003). Although not as economically important as FD, BN is present in most wine-producing regions of Europe. Its main vector is a planthopper, *Hyalestes obsoletus* (Boudon-Padieu, 2003). Reliable and sensitive methods of detection and differentiation of FD

*E-mail: matjaz.hren@nib.si

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from other GY-causing phytoplasmas are therefore required for efficient control of the disease.

Several methods for FD and BN detection have been developed:

(i) Early serological methods including ELISA and PCR-ELISA (Seddas *et al*., 1996; EPPO/CABI, 2003).

(ii) PCR-based methods including nested and multiplex-PCR that amplify either ribosomal or non-ribosomal phytoplasmic DNA (e.g. randomly cloned FD9 and Stol11 genomic fragments) (Daire *et al*., 1997; Lee *et al*., 1998; Clair *et al*., 2003).

(iii) RFLP methods using different restriction enzymes on ribosomal PCR products (Lee *et al*., 1998; Marzachi *et al*., 2001). The latter showed additional variability among FD phytoplasmas, distinguishing at least two subgroups of FD in Europe: 16SrV-C and 16SrV-D (Davis & Dally, 2001; Lee *et al*., 2004). RFLP on non-ribosomal PCR products distinguished three phytoplasma subgroups within FD (Angelini *et al*., 2001; Martini *et al*., 2002) and five subgroups within the stolbur group phytoplasmas associated with grapevine (Langer & Maixner, 2004).

(iv) More recently, real-time PCR based assays for universal phytoplasma detection (Christensen *et al*., 2004), as well as apple-proliferation-group-specific (Baric & Dalla-Via, 2004; Torres *et al*., 2005) and FD-specific (16SrV-C and 16SrV-D subgroup-specific) (Bianco *et al*., 2004) detection methods. Additionally, Galetto *et al*. (2005) developed real-time PCR assays for the specific detection of FD, BN and apple proliferation phytoplasma (SYBR® Green), and an assay for common detection of 16SrV, 16SrX and 16SrXII phytoplasma groups (TaqMan®).

In comparison to PCR/RFLP assays, real-time PCR methods may allow faster and more sensitive detection of phytoplasmas. Their levels of specificity and sensitivity can vary greatly depending on the real-time PCR chemistry chosen. For example, the SYBR® Green chemistry used by Galetto *et al*. (2005) is less specific than TaqMan®, which was used by Bianco *et al*. (2004) and in the common assay by Galetto *et al*. (2005). The use of TaqMan® MGB Probes (Minor Groove Binder Probes, Afonina *et al*., 1997) in this study greatly diminishes the possibility of detecting non-specific target DNA. Sensitivities of all published real-time PCR assays for detection of phytoplasmas were either not compared with the conventional phytoplasma detection (PCR/RFLP) or showed even lower sensitivity.

The aim of this work was to develop fast, reliable and sensitive real-time PCR detection systems based on TaqMan® MGB Probe chemistry for specific detection of BN and FD phytoplasmas, and for universal phytoplasma detection, which would enable enhanced diagnostic sensitivity and specificity compared to previously published detection systems. The approach included additional quality control steps to increase the confidence in results. Furthermore, a data-mining technique was used for classification and construction of decision trees in order to evaluate and compare the newly-developed real-time PCR and conventional PCR-based detection systems.

Materials and methods

Plant, insect and bacterial material

Phytoplasma strains for specificity testing were cultivated in *Catharanthus roseus* and were kindly provided by Dr R. Osler from the University of Udine, Italy. The strains included: elm yellows phytoplasma strain EY1 (EY1, 16SrV-A), aster yellows phytoplasma (AY, 16SrI-B), a stolbur phytoplasma isolate SE (SE, 16SrXII-A, original host: celery), Western X-disease transferred to *C. roseus* (WX, 16SrIII-A), apple proliferation phytoplasma strain AP15 (AP, 16SrX-A), pear decline phytoplasma (PD, 16SrX-C) and the European stonefruit yellows phytoplasma transferred to *C. roseus* from plum (ESFY, 16SrX-B). MA phytoplasma (16SrIII-B), originating from *Chrysanthemum leucanthemum* (Marguerite Daisy) cultivated on *Tanacetum cinerariifolium*, was also included in the study.

Several strains of plant pathogenic bacteria were obtained from the National Collection of Plant Pathogenic Bacteria (NCPPB), York, UK for specificity testing. These were: *Xylophilus ampelinus* (NCPPB 2217, type strain of the species), *Pseudomonas syringae* pv. *syringae* (NCPPB 281), *Erwinia amylovora* (NCPPB 683, type strain of the species) and *Clavibacter michiganensis* subsp. *sepedonicus* (NCPPB 4053). *Agrobacterium vitis* (KIS Av 13-2) was obtained from the collection of the Agricultural Institute of Slovenia, Ljubljana, Slovenia. Some of these strains are present in the grapevine microflora. Additionally, 29 bacterial isolates from plant extracts of various grapevine cultivars grown on nutrient agar (NA) (Bacto Nutrient Agar, Difco) or YPGA (yeast extract 7·0 g, proteose peptone 5·0 g, glucose 10·0 g, agar 15·0 g, distilled water 1 L, pH 7·0) were used. An additional five strains of grapevine pathogenic bacteria employed in this study were obtained from the collection of the Instituto Valenciano de Investigaciones Agrarias (Valencia, Spain) and were characterized as *Agrobacterium vitis*, *A. tumefaciens*, *A. rhizogenes*, *Xylella fastidiosa* Fetz and *X. fastidiosa* Staps.

Grapevine leaf samples were collected in the field from 148 grapevine plants (17 cultivars: Chardonnay, Beli Pinot, Sivi Pinot, Modri Pinot, Merlot, Refošk, Modra Frankinja, Sauvignon, Rebula, Šipon, Renski Rizling, Rumeni Muškat, Žametovka, Malvazija, Kerner, Zweigeld and Syrah). All the main coastal and continental wine-growing regions of Slovenia (Primorje, Posavje and Podravje) were represented. Three field-collected samples of field bindweed (*Convolvulus arvensis*) were also tested. Healthy field-collected grapevine plants were used as negative controls in real-time PCR reactions (cvs Barbera, Chardonnay, Sivi Pinot, Merlot and Refošk). The following sweep-captured insects were also tested: *Hyalesthes obsoletus* (four samples of at least 20 adults), *Scaphoideus titanus* (10 samples of at least 20 adults and one sample of at least 20 nymphs), *Euscelis incisus* (one sample of seven adults) and *Reptalus panzeri* (three samples of at least 20 adults). Sampling of grapevine plants was carried

out from July to October 2005. An additional 12 samples of field-collected apple trees (*Malus domestica*, leaf veins) that contained apple-proliferation-group phytoplasmas (16SrX) were tested. An additional 159 grapevine leaf samples (the same range of cultivars and geographical locations as in 2005) were collected from July to October 2006 and were analysed with real-time PCR only.

DNA samples from FD-infected (four samples) and BN-infected grapevine plants (one sample) were provided by Dr P. Ermacora of the University of Udine, Italy.

DNA extraction

The phytoplasma-enrichment procedure described by Ahrens & Seemüller (1992) was used, with some modifications; 1·5 g of leaf veins (whole leaves in the case of *C. roseus* and *C. arvensis*) were used as starting material, homogenized in 20 mL ice-cold grinding buffer and centrifuged for 5 min at 2500 ***g*** (4°C) followed by centrifugation of the supernatant for 25 min at 18 000 ***g*** (4°C). Lysis was performed in modified CTAB buffer (4% CTAB, 1% PVP MW 10 000, without 2-mercaptoethanol) at 65°C. After chloroform/isoamyl alcohol (24:1) extraction the DNA was precipitated with cold (–20°C) isopropanol, incubated at room temperature for 20 min and centrifuged for 15 min at 15 000 *g*. The pellet was then washed with cold 80% ethanol (–20°C), dried at room temperature and re-suspended in 50 μL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8·0) overnight at 4°C. Quantity and quality of DNA were analysed using gel electrophoresis (8 μL). The DNA concentrations of all phytoplasma and bacterial strains used in specificity testing and of several diagnostic samples (to estimate the range of concentrations) were measured spectrophotometrically with NanoDrop (NanoDrop Technologies). Tenfold dilutions of the samples were shown to be sufficient to keep the DNA load below 100 ng in real-time PCR reactions.

Conventional PCR assays

Each field-collected DNA sample from 2005 was tested for the presence of FD and BN using a set of PCR reactions:

(i) Specific detection of BN (Stol11 genomic fragment) using a STOL11f2/r1 primer pair (Daire *et al*., 1997) followed by a nested PCR reaction with STOL11f3/r2 (Clair *et al*., 2003);
(ii) Specific detection of FD (FD9 genomic fragment) using an FD9f/r primer pair (Daire *et al*., 1997) followed by a nested PCR reaction with FD9f3b/r2 (Clair *et al*., 2003); and
(iii) Using the universal primer pair F6/P7, which is actually a modified P1/P7 primer pair (Schneider *et al*., 1995): F6 is a modified P1 primer, shortened by two nucleotides at the 5′ and one nucleotide at the 3′ end (Dr P. Ermacora, University of Udine, Italy, personal communication). In the absence of a positive result, a nested PCR reaction with a U5/U3 primer pair (Lorenz *et al*., 1995) was performed on F6/P7 products.

All PCR assays were performed in 40-μL reactions with 2 μL of DNA (in the case of nested PCR reactions, original PCR products were diluted 100-fold) on a PCR System 9700 GeneAmp PCR cycler (Perkin Elmer). The manufacturer of all chemicals used in this study was Applied Biosystems. Reaction mixtures and conditions for amplification are summarized in Table 1.

Real-time PCR primers and assay design and setup

Publicly available phytoplasma sequences from the GenBank® database were aligned in VECTOR NTI software (Invitrogen) to find suitable regions for amplicon design. Primers and probes for the universal detection of phytoplasmas and for specific detection of BN and FD were then designed using PRIMER EXPRESS© (Applied Biosystems). TaqMan® MGB probes were labelled with 6–carboxyfluorescein (FAM) at the 5′ end and a non-fluorescent quencher (NFQ) with minor groove binder (MGB) at the 3′ end (Table 2). The specificities of the designed amplicons were tested *in silico* with a Basic Local Alignment Search Tool (BLAST) search of public databases.

All real-time PCR reactions were performed on an ABI PRISM® 7900 HT Sequence Detection System (Applied Biosystems) in optical 384-well plates with optical adhesive covers (both Applied Biosystems) using universal cycling conditions (2 min at 50°C, 10 min at 95°C,

Table 1 Amplification conditions of conventional PCR assays for phytoplasma detection

	Concentrations				Amplification [time(s)/T(°C))					
Primer pair	Pol[a] (U)	Primers (nM)	dNTPs (μM)	$MgCl_2$ (mM)	ID[b]	Amplification cycle			N[c]	FE[d]
STOL11f2/r1	2	375	62·5	1	90/92	30/92	30/55	50/72	40	600/72
FD9f/r	2	375	62·5	1	90/92	30/92	30/54	75/72	40	600/72
STOL11f3/r2 and FD9f3b/r2	2	375	300	2·5	90/92	40/92	40/55	70/72	30	600/72
6F/P7	1·6	400	150	2·5	120/92	30/93	30/50	90/72	40	600/72
U5/U3	2	375	37·5	1	75/92	30/93	30/57	50/72	35	600/72

[a]AmpliTaq® DNA polymerase.
[b]Initial denaturation.
[c]Number of cycles.
[d]Final elongation.

Table 2 Primers and MGB probes for Flavescence dorée (FD)-specific (FDgen), Bois noir (BN)-specific (BNgen) and universal phytoplasma (UniRNA) amplicons

Name	Orientation	Sequence (5′–3′)	Amplicon length	Location
UniRNA	Forward	AAA TAT AGT GGA GGT TAT CAG GGA TAC AG	73 bp	16S rRNA
	Reverse	AAC CTA ACA TCT CAC GAC ACG AAC T		
	Probe	5′FAM-ACG ACA ACC ATG CAC CA-3′NFQ[a]		
FDgen	Forward	TTA TGC CTT ATG TTA CTG CTT CTA TTG TTA	85 bp	sec Y gene
	Reverse	TCT CCT TGT TCT TGC CAT TCT TT		
	Probe	5′FAM-ACC TTT TGA CTC AAT TGA-3′NFQ[a]		
BNgen	Forward	AAG CAG GTT TAG CGA TGG TTG T	71 bp	Stol11 genomic fragment
	Reverse	TGG TAC CGT TGC TTC ATC ATT T		
	Probe	5′FAM-TTA ATA CCA CCT TCA GGA AA-3′NFQ[a]		

[a]NFQ, non-fluorescent MGB quencher.

followed by 45 cycles of 15 s at 95°C and 1 min at 60°C, with 9600 Emulation mode) which allowed running of all reactions on the same plate. Real-time PCR was performed in a final reaction volume of 10 μL containing 2 μL of sample DNA, 900 nM primers, 250 nM probe and 1× TaqMan® Universal PCR Master Mix (Applied Biosystems), which includes ROX™ as a passive reference dye. Each sample DNA was tested with all three amplicons for phytoplasmas and an amplicon for an endogenous control (in separate real-time PCR reactions): cytochrome oxidase (COX) for plants (Weller *et al*., 2000) or 18S rRNA for insects (eukaryotic 18S rRNA TaqMan endogenous control, Applied Biosystems). Endogenous controls amplified plant or insect DNA co-extracted with phytoplasmic DNA. All reactions were performed in two replicate wells in two dilutions (10- and 100-fold) on the same 384-well plate. An automated liquid handling system (Multiprobe® II PLUS EX, PerkinElmer) was used for pipetting a large number of DNA samples and master mixes onto the 384-well plates.

The software SDS 2·2 (Applied Biosystems) was used for fluorescence acquisition and calculation of threshold cycles (Ct). For this calculation, the baseline was automatically set and the fluorescent threshold was set manually to intersect with the linear part of amplification curves of all amplicons, resulting in the final Ct value for each well.

Specificity of amplicons

The specificity of all three real-time PCR amplicons was determined by testing the cross-reactivity with: (i) several phytoplasma strains cultivated in *C. roseus*; (ii) plant DNA extracted from leaf veins of several cultivars of symptomless field-grown grapevine plants; and (iii) several bacterial strains that can be present as epiphytes or saprophytes on grapevine plants and their tissues. For five of the bacterial isolates, DNA extracted from pure cultures was used (four from NCPPB and one from the Agricultural Institute of Slovenia). For 34 other isolates from grapevine plants, pure cultures grown on NA or YPGA were transferred to 100 μL of water and used in colony real-time PCR.

Real-time PCR products were visualized under UV light on 10% polyacrylamide gel.

Efficiency, limit of detection (LOD) and sensitivity of amplicons

Elm yellows phytoplasma strain EY1 is an American isolate of the elm yellows phytoplasma ('*Ca*. Phytoplasma ulmi'). It was used to develop the real-time PCR tests because it has the same target sequence as FD (Figs 1 and 3) and because no FD isolate was available.

Limit of detection (LOD) for all three real-time PCR amplicons was performed on 10-fold serial dilutions of EY1 DNA (340 ng μL^{-1}) and SE DNA (830 ng μL^{-1}) in water ranging from undiluted DNA to a 10^9-fold dilution factor. Five replicate wells per dilution were used. The slope (k) of the linear regression line between logarithmic values of relative DNA concentrations (y-axis) and Ct values (x-axis) was used to calculate the amplification efficiency, $E = (10^{[-1/k]})-1$, where a value of one corresponded to 100% amplification efficiency (Pfaffl, 2001). The squared regression coefficient after the linear regression (R^2) was also determined. Dynamic range, i.e. the range of concentrations for which Ct values were in linear relationship with logarithms of concentrations and range of detection, were also determined.

Sensitivities of PCR and real-time PCR were compared on EY1 and SE dilution series. Each dilution series was prepared by mixing the diluted phytoplasmic DNA with undiluted DNA isolated from leaf veins of several healthy grapevine plants. This healthy grapevine material had already been tested with real-time PCR and confirmed to be BN- and FD-negative. Thus, PCR sensitivity was evaluated for potential effects of host-material inhibition. Two separately prepared sets of dilution series were used, employing the same EY1 and SE DNA, but adding these to a different pool of DNA from healthy grapevine leaf veins. The concentration of DNA in both pools was 300–400 ng μL^{-1}.

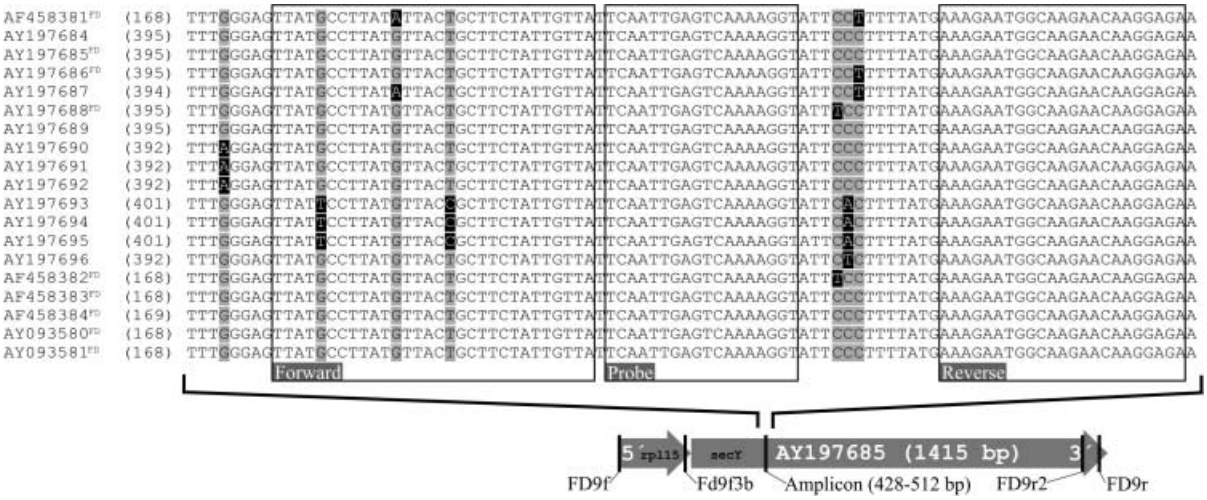

Figure 1 Schematic representation of the FDgen amplicon within aligned nucleotide sequences of the rpl15/secY gene (AY197685, FD9 fragment) obtained from GenBank. Accession numbers marked with suffix FD are Flavescence dorée phytoplasma sequences; the rest are other strains from the elm yellows group (16SrV). AY197690 is isolate EY1. The position of primers for detection of FD with conventional PCR FD9f/r (Daire *et al.*, 1997) and FD9f3b/r2 (Clair *et al.*, 2003) are also shown in AY197685.

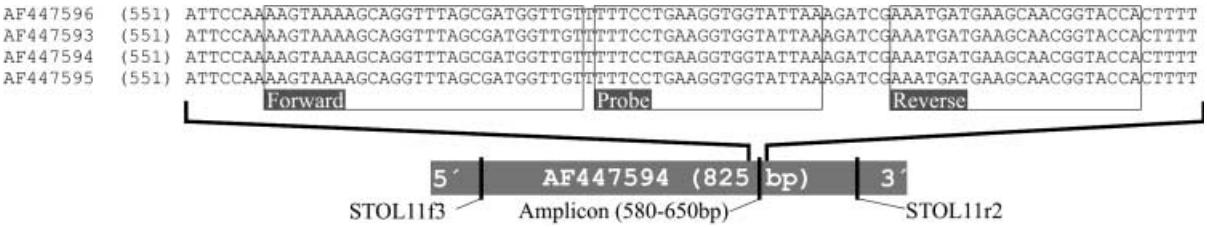

Figure 2 Schematic representation of the BNgen amplicon within the aligned nucleotide sequences of the Stol11 fragment (AF447594) obtained from GenBank. The position of the nested PCR primer pair STOL11f3/r2 (Clair *et al.*, 2003) for detection of Bois noir phytoplasma are shown in AF447594. The STOL11f2/r1 primers (Daire *et al.*, 1997) are situated outside the sequence shown in the figure.

Data mining approach

Decision trees were generated using the WEKA machine learning workbench (Frank *et al.*, 2004) and its algorithm J48, which is WEKA's implementation of the C4·5 algorithm. As described in Larose (2005), the algorithm visits each possible decision node, in this case all conducted assays, selecting the optimal split, until no further splits are possible. Decision trees were drawn for the PCR-based detection system and the newly-developed real-time PCR assays, and evaluated using 10-fold cross validation. The percentage of correctly classified instances was calculated for both methods (PCR and real-time PCR).

Diagnostic sensitivity and specificity were also calculated for evaluation of both detection systems according to Dinnes *et al.* (2005). Diagnostic sensitivity (proportion of diseased samples that gave positive results) was calculated as true positives/total diseased and diagnostic specificity (proportion of non-diseased samples that gave negative test results) as true negatives/total non-diseased. Results from all three amplicons were used for calculations.

Results

Design of real-time PCR assay

All amplicons were designed using the nucleotide sequences available in GenBank®. Schematic representations of each amplicon within the aligned nucleotide sequences used for amplicon design, including their GenBank accession numbers, are presented in Figs 1–3 and Table 2. The FDgen amplicon was designed within the *SecY* gene which codes for a translocation protein (GenBank Accession No. AY197685) using nucleotide sequences of several Flavescence dorée phytoplasma isolates including FD-C (AF458382, AY197688), FD-D (AY197685), FD70 (AF458383, AY197686), FD92 (AF458384) – similar to FD-D (AY197685), FD2000 (AY093581) and HD1 (AF458381). This amplicon is situated within the primer pairs FD9f/r (Daire *et al.*, 1997) and FD9f3b/r2 (Clair *et al.*, 2003) previously used for detection of FD, and contains two polymorphic nucleotides positioned between the 3′ end of the MGB probe and the reverse primer, which therefore do not affect the specificity of the

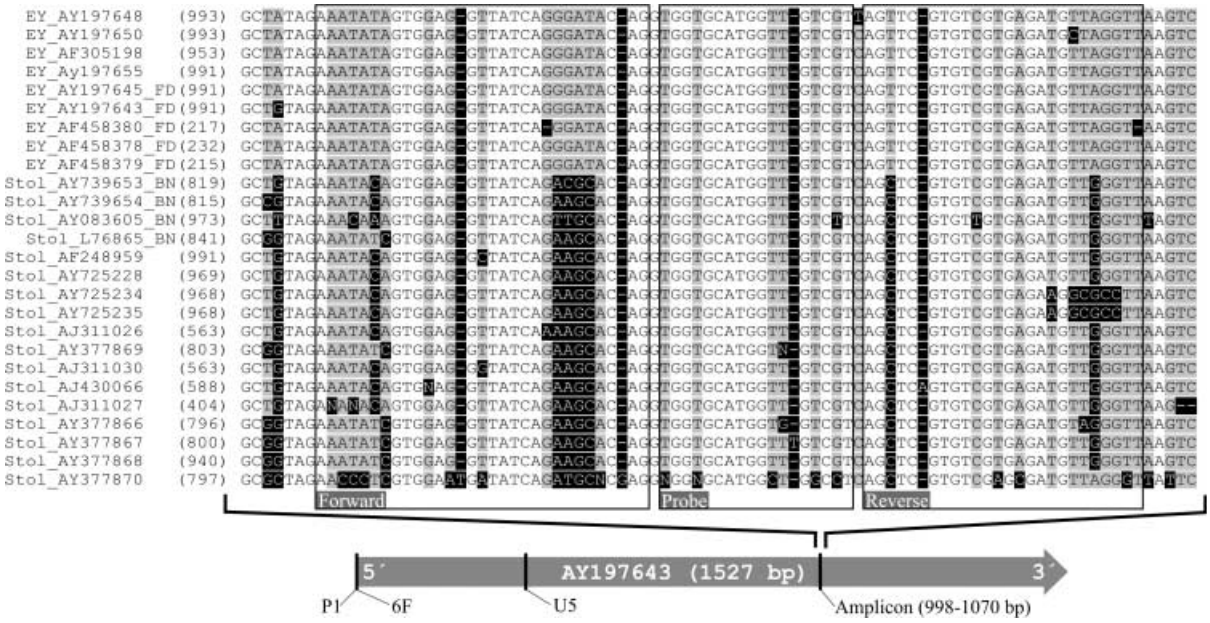

Figure 3 Schematic representation of the UniRNA amplicon within the aligned nucleotide sequences of the 16S rRNA gene (AY197643) obtained from GenBank. Sequences belonging to the elm yellows group (16SrV) are marked with the prefix EY and those marked by the suffix FD are Flavescence dorée phytoplasmas. AY197655 is isolate EY1. Sequences belonging to the stolbur group (16SrXII) are marked with the prefix Stol and those marked with the suffix BN belong to Bois noir phytoplasmas isolated from grapevine. Positions of universal PCR primers for detection of phytoplasmas, P1 (Schneider *et al.*, 1995), 6F (modified P1 primer, Dr P. Ermacora, University of Udine, personal communication) and U5 (Lorenz *et al.*, 1995) are shown in AY197643 (the corresponding reverse primers are situated outside the sequence shown). Thirty-five additional sequences of the 16 rDNA gene were included in the alignment, but are not shown in the figure because they were identical to at least one of the sequences already present.

amplicon. An additional polymorphic nucleotide appears in the forward primer, but only in the isolate HD1 and at the 5′ end of the primer, which should not substantially affect the annealing properties (Fig. 1). The BNgen amplicon was designed within the Stol11 genomic fragment (AF447594) using nucleotide sequences that showed no polymorphism within the amplicon region. It is situated within the primer pairs STOL11f2/r1 (Daire *et al.*, 1997) and STOL11f3/r2 (Clair *et al.*, 2003), which were previously used for detection of BN (Fig. 2).

The universal amplicon UniRNA was initially designed to amplify the FD-specific region of the 16S rRNA. Although a high number of ribosomal sequences from the stolbur (16SrXII) and elm yellows (16SrV) groups were used in the design process, the number of polymorphic nucleotides between those two groups was not high enough to even allow the specific discrimination of group 16SrV (Fig. 3). The universal amplicon UniRNA amplifies FD and BN phytoplasmas (along with other phytoplasma strains), but with a distinct preference for FD. Therefore it was included in the protocol as a tool to increase the sensitivity of FD detection and other phytoplasma types. *In silico* testing of specificity of all three amplicons using a BLAST search of public databases showed no unspecific hits with high similarity to the amplicon sequences ($E > 0{\cdot}011$).

Performance characteristics of real-time PCR assays

Performance characteristics were determined by amplifying serial dilutions of phytoplasma-infected plant DNA in water (Table 3). Phytoplasma-infected *C. roseus* plants were used as a source of phytoplasma because of their high phytoplasma content. All designed amplicons show a broad dynamic range (at least five log orders of magnitude) and were very sensitive. High variability in Ct values observed in the last dilution, still giving positive results, was attributed to the stochastic effect in target copy distribution in replicate reactions. The occurrence of such stochastic effects in real-time PCR reactions is considered to occur when less than 10 copies of the target are present in the reaction (Morrison *et al.*, 1998; Ellison *et al.*, 2006). Therefore, it was concluded that the limit of detection was below 10 copies per reaction. Intra-run reproducibility was very high for all three amplicons as coefficients of variations of Ct values (cv, results not shown) between the parallels did not exceed 10% when the amplicon copy number was within the dynamic range of the method. The efficiency of amplification was above 89%. Parameters were also evaluated for the serial dilutions of phytoplasmic DNA mixed with grapevine DNA isolated from healthy, phytoplasma-uninfected grapevine leaves to imitate real grapevine samples (results from the first set of the dilution series are shown). No significant

Table 3 Performance characteristics of real-time PCR measured for all three amplicons used in this study in water dilutions and for the FDgen and BNgen amplicons in dilutions prepared with phytoplasma-negative grapevine DNA isolated from healthy grapevine leaf veins (approximately 100 ng of genomic DNA per reaction in undiluted samples)

Amplicon	Target	Range of detection (dilution factors)	Dynamic range (dilution factors)	Linear regression			LOD[b] (average Ct)
				Slope (k)[a]	R^2	E	
UniRNA	EY1 (water)	$1–10^7$	$1–10^7$	–3·3179	0·9967	1·00	35·4
UniRNA	SE (water)	$1–10^7$	$1–10^6$	–3·5067	0·9282	0·93	34·1
FDgen	EY1 (water)	$1–10^6$	$1–10^6$	–3·4061	0·9959	0·97	35·1
BNgen	SE (water)	$*10–10^7$	$*10–10^6$	–3·4745	0·9937	0·94	35·9
FDgen	EY1 (plant DNA)	$10–10^6$	$10–10^6$	–3·6291	0·9962	0·89	37·3
BNgen	SE (plant DNA)	$10–10^6$	$10–10^6$	–3·2864	0·9983	1·02	34·6

*Amount of DNA in undiluted sample was too high, which probably inhibited the detection of SE.
[a]k = slope of the linear regression line in a plot of Ct values against log of relative concentrations; R^2 = average square regression coefficient; E = efficiency of amplification.
[b]LOD = limit of detection.

Table 4 Specificity of the real-time PCR assay for phytoplasma detection

Host	DNA from organism (strain)	Average Ct value of 10-fold dilutions (*c.* 50–100 ng genomic DNA)		
		UniRNA	FDgen	BNgen
Catharanthus roseus	EY1 phytoplasma isolate (16SrV-A, group reference strain)	15·4	15·0	ND
	Stolbur SE phytoplasma (16SrXII)	26·9	ND	16·6
	AY phytoplasma (16SrI)	27·3	ND	ND
	WX-disease phytoplasma (16SrIII)	21·0	ND	ND
	AP phytoplasma (16SrX)	19·9	ND	ND
	PD phytoplasma (16SrX)	14·5	ND	ND
	ESFY phytoplasma (16SrX)	18·8	ND	ND
	MA phytoplasma (16S III-B)	28·5	ND	ND
Bacterial isolates	*Xylophilus ampelinus* (NCPPB 2217[T])	ND	ND	ND
	Agrobacterium vitis (KIS Av 13-2)	ND	ND	ND
	Pseudomonas syringae pv. *syringae* (NCPPB 281)	ND	ND	ND
	Clavibacter michiganensis subsp. *sepedonicus* (NCPPB 4053)	ND	ND	ND
	Erwinia amylovora (NCPPB 683[T])	ND	ND	ND
Grapevine	Healthy *Vitis vinifera* (cvs Barbera, Chardonnay, Sivi Pinot, Merlot and Refošk)	ND	ND	ND
	BN-infected grapevine plants (range of Ct values from all BN-positive samples)	> 29·7	ND	21·7–37·4
	FD-infected grapevine plants (range of Ct values from all FD-positive samples)	19·3–32·0	21·8–34·9	ND
Scaphoidens titanus (phytoplasma-free)		ND	ND	ND
Reptalus panzeni (phytoplasma-free)		ND	ND	ND

[T]Type strain for the species.
ND, not detected.

effects on sensitivity, limit of detection, repeatability of the method or efficiency of amplification were observed.

Real-time PCR products were visualized on a 10% polyacrilamide gel stained with ethidium bromide. Single bands of expected size in samples containing phytoplasmic DNA confirmed the absence of unspecific amplification (data not shown).

Specificity of real-time PCR phytoplasma detection

The universal amplicon (UniRNA) successfully amplified all phytoplasma strains, albeit with different sensitivities. The FD-specific amplicon (FDgen) amplified only elm yellows phytoplasma EY1. The BN-specific amplicon (BNgen) successfully amplified only stolbur phytoplasma SE. No cross-reactivity with DNA of healthy grapevine plants was observed (Table 4). Additionally, several grapevine epiphytic bacterial strains were isolated and tested for cross-reactivity with these amplicons. Only one out of 34 bacterial isolates gave a weak signal with the UniRNA amplicon (Ct values above 34·2) at more than 10^5 genome copies loaded per reaction, meaning that these results are unlikely to confound real diagnostic tests.

Sensitivity of real-time PCR compared to conventional PCR

Artificial samples imitating infected grapevine samples were prepared by serial dilutions of EY1 and SE phytoplasmic DNA mixed with healthy grapevine DNA.

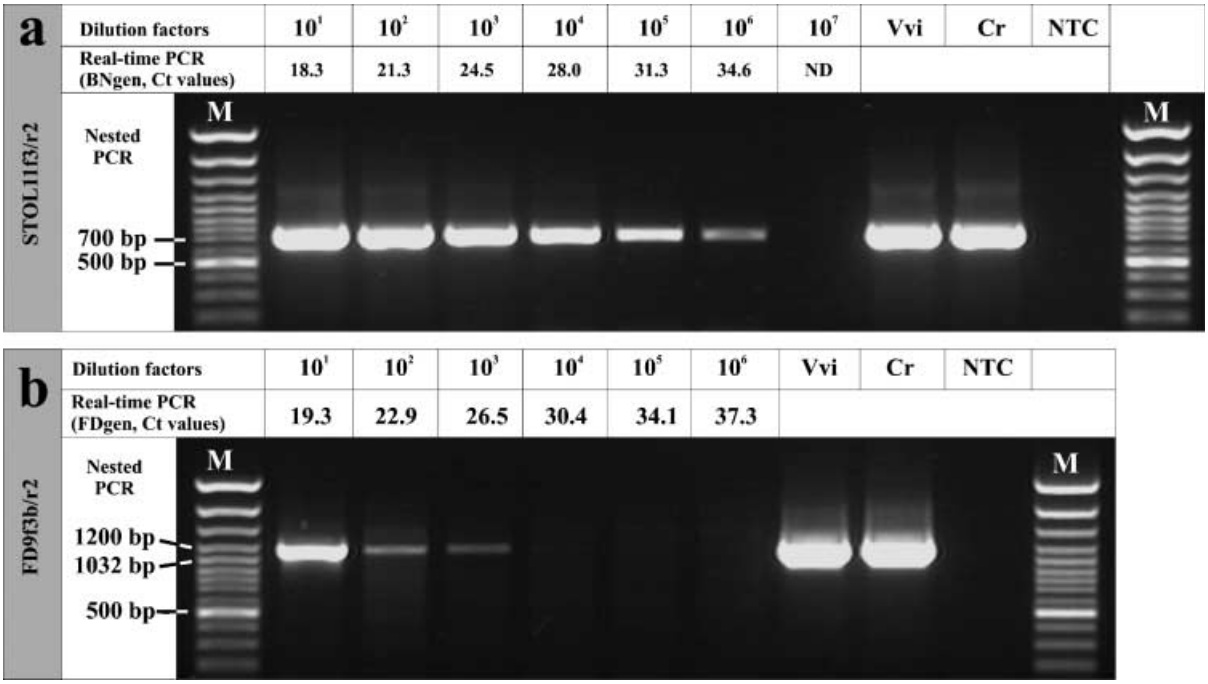

Figure 4 (a) Nested PCR (STOL11f3/r2) products and results of real-time PCR (BNgen) of healthy grapevine DNA spiked with SE (stolbur) phytoplasmic DNA (10–10^7-fold dilution). Length of PCR products is 720 bp. Results of the first set of dilution series are shown. (b) Nested PCR (FD9f3b/r2) products and results of real-time PCR (FDgen) of healthy grapevine DNA spiked with EY1 phytoplasmic DNA 10–10^6-fold dilution). Length of PCR products is 1160 bp. Average Ct values of real-time PCR for the corresponding dilution factor are also shown. M = GeneRuler™ 100-bp DNA Ladder Plus (Fermentas); Vvi = a grapevine sample infected with Bois noir (BN) (a) or Flavescence dorée (FD) phytoplasma (b); Cr = DNA isolated from *Catharanthus roseus* that contains a high concentration of SE phytoplasma DNA (a) or EY1 phytoplasma DNA (b); NTC = no-template control; ND = not detected. Vvi and Cr, positive controls.

Detection of phytoplasmas by conventional nested PCR and real-time PCR was performed on two independent sets of artificial samples. SE was detected with the same level of sensitivity by both conventional nested PCR and the real-time PCR assay with the BNgen amplicon, the last signal being observed at the 10^6-fold dilution factor (weak band on agarose gel and average Ct value of 34·6 in the first and 35·8 in the second set, Fig. 4a). In the case of EY1, however, real-time PCR with the FDgen amplicon showed a similar sensitivity to that with the BNgen amplicon, with the limit of detection occurring at a 10^6-fold dilution factor (the last weak band on agarose gel was observed at an average Ct value of 37·3 in the first and 35·5 in the second set at this 10^6-fold dilution factor; Fig. 4b). Conventional nested PCR showed the final signal at the 10^3-fold dilution level in the first set (Fig. 4b) and at the 10^7-fold dilution level in the second set (results not shown).

Comparison of real-time PCR detection system and conventional PCR on field samples

A total of 153 field grapevine samples collected in 2005 were analysed for the presence of different phytoplasmas, using both conventional nested PCR and real-time PCR detection systems. A detailed comparison of the PCR vs. real-time PCR results is shown in Table 5.

In addition, three field bindweed and 19 insect samples were analysed. Two BN-positive bindweed samples by conventional PCR were also detected as BN-positive with real-time PCR. The third sample was only positive with nested PCR (conventional) or in the UniRNA assay (real-time). Further analysis of U5/3 PCR products by RFLP confirmed BN infection (data not shown). All five BN-positive insect samples were confirmed with real-time PCR (three samples of *H. obsoletus*, one sample of *R. panzeri* and one sample of *E. incisus*) and one additional BN-positive sample (*H. obsoletus*) was discovered among the 13 PCR-negative insect samples.

The results show that the real-time PCR phytoplasma detection system developed was even more sensitive than conventional PCR methods, as additional phytoplasma positives were confirmed in grapevine plants (14/38, 13 samples showed weak symptoms and one lacked all disease features) and in one insect sample. Ranges of Ct values (Table 6) obtained in different experiments showed that the concentration of phytoplasma could vary more than 100-fold from sample to sample. This was true for samples of grapevine leaves, insect vectors and field bindweed.

Ct values for UniRNA showed that the amplicon detected FD and BN with different sensitivities. In the case of an FD-positive sample, FDgen Ct values should be at least two cycles below UniRNA Ct values and in the case

Table 5 Comparison of real-time PCR and conventional PCR detection of phytoplasmas in 153 field samples of grapevine collected in 2005

		Conventional PCR					
		NEG[a]	BN+	FD+	BN+FD+[b]	Undet.[c]	Total
Real-time PCR (MGB)	NEG[a]	24				2	26
	FD+			9	1		10
	BN+	10	99			1	110
	BN+FD+[b]				3		3
	Undet.[c]	4					4
	Total	38	99	9	4	3	153

[a]NEG = phytoplasma-negative samples.
[b]BN+FD+ = mixed infections (result of pooling of several BN- and FD-infected samples in the field).
[c]Undet. = samples with undetermined phytoplasma type (non-BN and non-FD type).

Table 6 Range of Ct values for PCR phytoplasma detection in various sample types using different amplicons. Bois noir (BN)-positive and Flavescence dorée (FD)-positive samples were classified according to conventional PCR results. Ct values are given for 10-fold sample dilutions. Mixed FD/BN infections (four samples) were counted both as FD-positive and BN-positive samples

Real-time PCR amplicon	BN positive samples			FD positive samples	Healthy samples	
	grapevine	insects	field bindweed	grapevine	grapevine	insects
BNgen	21·7–37·4	20·9–38·2	18·9–37·7	ND[b]	ND	ND
FDgen	ND	ND	ND	21·8–34·9	ND	ND
UniRNA	> 29·7	29·8–39·2	> 28·3	19·3–32·0	ND	ND
COX/18SrRNA[a]	16·9–24·9	15·1–20·7	17·5–18·7	18·6–21·2	17·5–24·8	18·9–38·3

[a]18S rRNA amplicon was used as the endogenous control for insect samples and COX for plant samples.
[b]Not detected.

of a BN-positive sample, BNgen Ct values should be at least four cycles above UniRNA Ct values. If these differences in Ct values deviate from this rule it can concluded that an additional type of phytoplasma is present in the sample. Thus, the presence of another phytoplasma type was confirmed in three BN-positive samples.

The difference in Ct values between 10-fold and 100-fold dilutions (ΔCt value) was monitored for each amplicon. As no inhibition was detected whilst using the protocols described in this paper (results not shown), the use of only 10-fold diluted DNA is proposed. When introducing new DNA extraction methods, ΔCt should, however, be monitored to assess the inhibition and to avoid false negative results caused by the inhibition of amplification.

Twelve samples of apple trees infected with phytoplasma belonging to the apple proliferation group (16SrX) also tested positive with the UniRNA amplicon, with Ct values ranging from 19·6 to 36·5. Grapevine samples collected in 2006 were analysed only with the real-time PCR detection system and produced the following results: 45 samples phytoplasma-negative, 96 samples BN-positive, 12 FD-positive and 6 phytoplasma positive containing an undetermined phytoplasma type (non-BN and non-FD).

Data mining

A data-mining approach was used to produce decision trees for conventional PCR and newly developed real-time PCR detection systems for grapevine samples (Fig. 5a,b). The algorithm produced decision trees that showed which tests were crucial and used cross-validation to calculate how many samples (instances) were classified correctly/incorrectly for each result option (numbers in brackets) and to calculate the overall percentage of correctly classified samples. These results could be used to estimate which detection system performed better. The decision trees also offered a graphic overview over the complexity of the detection system and could be used to make the detection system more efficient. It should be noted, however, that the schemes did not represent the actual experimental design but only assisted the interpretation of results.

The decision tree in Fig. 5a was generated using test results from the conventional PCR detection system (samples collected in 2005). It showed that four different PCR tests would have to be used for classification of an unknown sample. Apart from specific primer pairs for nested PCR-FD9 (FDpf3b/r2), PCR-Stol11 (STOL11f2/r1) and nested PCR-Stol11 (STOL11f3/r2), the primer pair for universal detection of phytoplasmas, U5/3, was also shown to be vital for the correct classification of samples. Because nested PCR-FD9 and U5/3 required a preceding PCR step, the number of required PCR assays was increased to six (all PCR assays used in this study). Tenfold cross-validation showed that the proposed decision tree for PCR assays correctly classified 93·9% of the samples tested and that tests leading to BN-positive results were the most problematic (Fig. 5a).

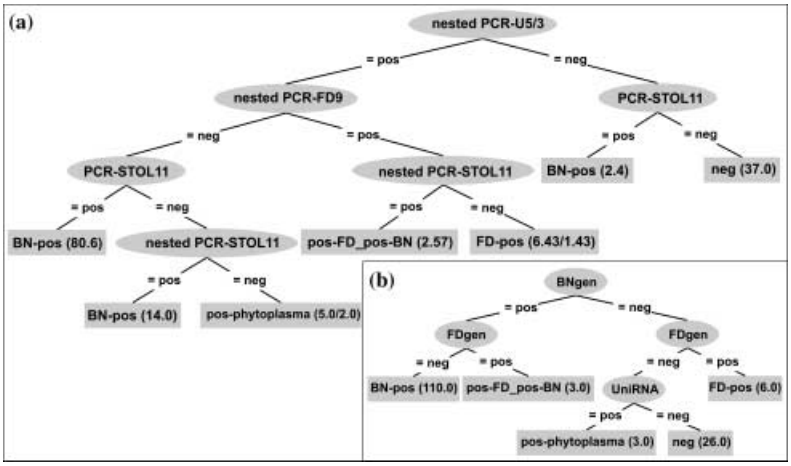

Figure 5 Decision trees for (a) PCR assays and (b) real-time PCR assays. Decision nodes that were shown to be important for classification of samples are shown in ellipse while leaf nodes, showing the result of the assay, are shown in rectangle shapes: BN-pos = Bois noir-positive; FD-pos = Flavescence dorée-positive; pos-FD_pos-BN = FD- and BN-positive (mixed infection); pos-phytoplasma = unclassified phytoplasma; neg = phytoplasma negative. Numbers in brackets denote the number of cases for which the proposed pathway was correct/incorrect. The results of the tests (= pos or = neg) are shown in branches.

The decision tree in Fig. 5b was generated using real-time PCR results on the same samples. It was simpler than the decision tree for conventional PCR and showed that all three real-time PCR amplicons were needed for classification of unknown samples. The fact that UniRNA was not positioned at the top of the decision tree reflects its unequal sensitivity towards FD and BN phytoplasmas. Tenfold cross-validation showed that the decision tree for real-time PCR assays correctly classified 100% of the samples. When an additional 156 grapevine samples collected in 2006 were included in the dataset for analysis with data mining, the same percentage of correctly classified samples was calculated (100%).

Diagnostic sensitivity and specificity were also used to evaluate both detection systems on samples collected in 2005. Both parameters supported the results from the data-mining approach, i.e. favouring the real-time PCR detection system: the diagnostic sensitivity of conventional PCR compared to real-time PCR was 88·0% and diagnostic specificity 92·3%.

Discussion

Diagnosis of GY is currently predominantly based on PCR and RFLP analysis. Although real-time PCR for detection of GY has already been reported (Bianco *et al*., 2004; Galetto *et al*., 2005), a complete system for the specific detection of GY with similar or better sensitivity than conventional PCR/RFLP had not yet been made available. This paper reports TaqMan® MGB-based phytoplasma detection systems targeting ribosomal and non-ribosomal phytoplasma genes.

Quality and quantity of isolated DNA greatly influence the detection of GY. Monitoring the Ct values of endogenous controls (COX) allows the estimation of both parameters, therefore increasing the confidence in results. In this way each reaction with a low amount of DNA can be identified. Ct values for COX in the DNA extraction procedure used in this research work varied from 17 to 25. Higher Ct values were an indicator of lower amounts of plant DNA and presumably lower amounts of phytoplasmic DNA present in the reaction, which therefore decreased the confidence in results. Higher COX Ct values indicate that the results are not reliable and the DNA for that sample should be re-extracted and re-tested. 18S rRNA was used in the same way with insect samples. Ct values reached as low as 15·1 and depended greatly on the number of insects used for DNA extraction.

Despite the comparable levels of sensitivity assessed in evaluation of each assay on artificial samples, the analysis of field-collected grapevine samples showed significantly higher diagnostic sensitivity of real-time PCR in comparison to conventional PCR by revealing some false negative PCR results. Ten out of 38 samples that were detected as phytoplasma-negative by PCR were shown to be BN-positive by real-time PCR. All 10 samples showed weak symptoms, thereby eliminating the possibility of false positive real-time PCR results. Additionally, four out of 38 PCR-negative samples were phytoplasma-positive by real-time PCR, i.e. the presence of an undetermined type of phytoplasma was confirmed (only the UniRNA amplicon produced a signal). The increased sensitivity of real-time PCR can be partly attributed to the short regions of target DNA that are amplified in TaqMan® MGB probe-based real-time PCR assays (at least 10 times shorter than conventional PCR and approximately 50% shorter that TaqMan® amplicons). Therefore real-time PCR reactions might be less sensitive to DNA degradation or DNA-binding inhibitors present in the samples.

The robustness of the real-time detection system was additionally confirmed with the field-collected grapevine samples in the following ways: (i) in the consistent ability to amplify GY phytoplasma in field-collected grapevine from various geographical locations in Slovenia and Italy throughout the sampling season (July–October) and (ii) in the ability to confirm negative results of conventional PCR (healthy samples) without producing false positive signals, despite the seasonal variation of epiphytic microflora on

grapevine plants. Although one of 34 bacterial isolates from grapevine microflora was detected with high Ct values with the UniRNA amplicon, the 10^5 bacterial genome copies per reaction is a situation highly unlikely to occur in grapevine samples.

Based on the results from a complete sampling season, the following approach is proposed for GY detection with real-time PCR using 10-μL reactions (2 μL DNA per well) on 384-well plates. Each sample (10-fold diluted DNA) should be tested with the following amplicons in three replicate wells: BNgen, FDgen, UniRNA and COX or 18S rRNA. UniRNA should be used to confirm the presence of FD and BN phytoplasmas and to better analyse mixed BN/FD infections. In the case of FD-only positive samples, Ct values for UniRNA should be two to three cycles below the FDgen Ct values. In the case of BN-only positive samples, the Ct values for the UniRNA amplicon should be more than four cycles higher than for BNgen. This differential response of UniRNA towards BN/FD is a result of higher specificity of the amplicon for the FD phytoplasma (Fig. 3). For three BN-positive samples the Ct values for UniRNA were higher than for BNgen, indicating the possibility of the presence of another type of phytoplasma alongside BN. When only the UniRNA amplicon produced a signal, the sample was considered to be infected with an undetermined phytoplasma type. In all such cases (four grapevine samples) Ct values were high (above 30), even in undiluted DNA, and the plants showed only weak symptoms or lacked them entirely. Therefore, it must be conceded that FD might be present in a very small concentration as the FD-specific amplicon is approximately 10 times less sensitive than the universal one.

Decision rule building has been used in plant pathology diagnostics since the 1970s (Michalski & Chilausky, 1999). Decision trees are often used in other fields of diagnostics because they represent an easily understandable method for extracting important knowledge from data which would otherwise remain confusing because of a large number of samples and assays requiring analysis. A common approach when comparing two (or more) diagnostic methods is the calculation of diagnostic sensitivity and specificity (Dinnes *et al*., 2005) for each pair of the compared methods. Such an approach requires one diagnostic method to be set as the reference standard. On the other hand, data mining allows simultaneous comparison of two or more diagnostic methods without having to select one as the reference standard. Thus all prejudice about which method is the most suitable for the reference standard is excluded. From decision trees for the PCR-based detection system and the newly-developed real-time PCR assays (Fig. 5a,b) it can be seen that three assays with real-time PCR produced a more accurate classification (100% correctly classified samples) than did six conventional PCR assays (93·9% correctly classified samples). When real-time PCR data from 307 grapevine samples collected in 2005 and 2006 were used in data mining the percentage of correctly classified samples remained 100%, again confirming the robustness of the real-time PCR detection system.

In summary, a method has been developed that has a better sensitivity and robustness than conventional PCR detection of GY and provides additional information about each sample that enhances the reliability of results. Results can also be interpreted in a quantitative way (e.g. with a relative quantification approach) if such data are needed for the study of epidemiology or physiology of the disease. Furthermore, the new method is substantially less laborious and time consuming.

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