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Panel of 23S rRNA Gene-Based Real-Time PCR Assays for Improved Universal and Group-Specific Detection of Phytoplasmas▽†

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Primers and probes based on the 23S rRNA gene have been utilized to design a range of real-time PCR assays for routine phytoplasma diagnostics. These assays have been authenticated as phytoplasma specific and shown to be at least as sensitive as nested PCR. A universal assay to detect all phytoplasmas has been developed, along with a multiplex assay to discriminate 16SrI group phytoplasmas from members of all of the other 16Sr groups. Assays for the 16SrII, 16SrIV, and 16SrXII groups have also been developed to confirm that the 23S rRNA gene can be used to design group-specific assays.

Phytoplasmas are wall-less bacteria of the class *Mollicutes* that inhabit plant phloem and insect vectors. They are known to cause disease in hundreds of plant species worldwide and are classified into 16Sr groups and "*Candidatus* Phytoplasma" species based on their 16S rRNA gene sequences (14, 21). Since it is not possible to isolate and study phytoplasmas in pure cultures, conventional PCR has become the method of choice for detection and diagnosis. Numerous PCR primer combinations have been devised to amplify the 16S rRNA gene for diagnostics; some of these are universal primers that work on DNA from all phytoplasma phylogenetic groups, while others are group specific (8, 10, 17). However, diagnostics based on these primers can be problematic, with occasional false positives through amplification of other bacteria that might be present in samples (11).

Real-time PCR assays have also been developed for both generic and specific phytoplasma detection. In general, the aims have been to produce very specific and sensitive assays for detection of a group-specific nature. For example, TaqMan and SYBR green chemistries have been applied in various diagnostic assays for 16SrX group (1, 3, 18), 16SrV and -XII group (2, 9, 15), 16SrI group (16, 20), and 16SrVI group (5) phytoplasmas. However, several of these specific assays cross-react with phytoplasmas from other groups (5, 16). The most successful attempt to develop a fully universal assay has been a TaqMan assay that was demonstrated to amplify all 16Sr groups except 16SrIV, -XIII, and -XIV, which were not tested (4).

Development of new universal and multiplex real-time PCR assays. The primers and probes used for most phytoplasma real-time PCR assays have been based on 16S rRNA gene sequences, although some attempts have been made to design them based on the *tuf* (20), nitroreductase (9), and *secY* (15) genes. Generally these assays have also employed the use of separate assays, such as the cytochrome *c* oxidase (COX) assay, as endogenous controls to detect plant or insect DNA, due to the high likelihood of PCR inhibitors in DNA extracts (2, 3, 4, 5, 15, 16). In previous work, we cloned and sequenced the first 500 bp of the 23S rRNA genes from a diverse range of phytoplasmas and showed that there is significant sequence variation between isolates (13). Real-time PCR primers and probes were therefore designed using these partial 23S rRNA gene sequences. The alignment used was described by Hodgetts et al. (13), with the addition of other publicly available phytoplasma sequences and sequences from a range of other bacteria (see Fig. S1 in the supplemental material for details). Primer express V2 (ABI) was used to design primers, along with a manual assessment of the proposed primers' binding action across all of the phytoplasma 16Sr groups. The primer and probe sequences and reporter/quencher dyes are listed in Table 1, and each of the assays' primer/probe combinations and their specificities are indicated in Table 2. All primers were synthesized by Eurofins MWG (Ebersberg, Germany) and probes by Applied Biosystems (California).

Real-time PCR was carried out with an ABI Prism 9700HT instrument, and data were analyzed with sequence detection system V or a Stratagene Mx3005P instrument in 96-well plates. In all cases, 1 μl of DNA extract (concentration as extracted at 0.5 to 1.0 mg ml^{-1}) was used in 24 μl of master mix, and all samples were tested in duplicate. Negative controls containing nuclease-free water in the place of DNA were included in all runs. Real-time PCR was carried out using TaqMan core reagents (Applied Biosystems) consisting of 1× buffer A (50 mM KCl, 10 mM Tris-HCl, pH 8.3, carboxy-X-rhodamine passive reference dye), 5.5 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate, and 0.625 U Ampli*Taq* Gold. All primers were used at a final concentration of 300 nM and all probes at a final concentration of 100 nM. Universal cycling conditions were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles each consisting of 15 s at 95°C and 1 min at 60°C. Results were analyzed in terms of the average cycle threshold (C_T) values (cycle that produces a positive PCR signal as determined by the algorithm built into the software). In order to check the reproducibility and repeatability of the assays, the assays were performed in two laboratories (CSL,

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TABLE 1. Characteristics of primers and TaqMan MGB probes

Primer or probe	Sequence (5′–3′)[a]	Reporter dye (5′)	Quencher dye (3′)
Primers			
JH-F 1	G**GT**CTCCGAATGGG**AA**AACC		
JH-F all	**ATT**TCCGAATGGG**GC**AACC		
JH-R	CTCGTCACTACTACC**R**GAATCGTTATTAC		
JH-F CSPW	CTACTACAATTACCTTGTAGTTTTTT**GATGG**		
JH-F LY	ACCTACTATAATAAACTTATAGTTTTTTTGAT**GT**TA**AAG**		
JH-F EAT	AACCTACTGCAATAAACTTGTAGTTTTTTT**AG**		
Probes			
JH-P 1	CGC**GGC**GAACTGAAAT	FAM	MGB-NFQ[c]
JH-P all	CGC**AGT**GAACTGAAAT	VIC	MGB-NFQ
JH-P uni	AACTGAAA**T**ATCTAAGTAA**C**	FAM	MGB-NFQ
JH-P 2	A**GGT**AA**C**T**A**ATT**GATTG**AAAGGA	TET[b]	MGB-NFQ
JH-P 12	**CATC**ATC**CTTGA**AGAAG**GA**	FAM	MGB-NFQ

[a] Positions that correspond to polymorphic sites in the sequences of phytoplasma isolates are shown in bold.
[b] TET, [TET] dye phosphoramidite.
[c] MGB-NFQ, molecular-groove-binding nonfluorescent quencher.

York, United Kingdom, and the University of Nottingham) with instruments from two different manufacturers (ABI and Stratagene, respectively). The COX assay (22), which amplifies from host plant DNA, was used on all samples to confirm the lack of PCR inhibitors.

Both the universal assay and the multiplex assay (Table 2) were evaluated using DNA from more than 29 phytoplasma isolates, representing all of the major 16Sr groups, 16SrI to -XIV, with the exception of 16SrVIII ("*Candidatus* Phytoplasma luffae"), where DNA was not available. The DNA was predominantly from infected *Catharanthus roseus* plants but also from napier grass (*Pennisetum purpureum*), Bermuda grass (*Cynodon* spp.), and coconut (*Cocos nucifera*). The phytoplasmas used are listed in Tables 3 and 4, and their origins and maintenance have been described previously (12, 13). Total DNA was extracted from small quantities (300 mg) of leaf tissue by use of the cetyl trimethyl ammonium bromide method of Doyle and Doyle (7), and all DNA extracts used in the quantitative PCR assay development stage were checked for the presence of PCR inhibitors by conventional PCR prior to use in real-time PCR. Where DNA samples exhibited PCR inhibition, they were cleaned up using polyvinylpolypyrrolidone (6).

TABLE 2. Combinations of primers and probes used for the different assays

Assay specificity	Forward primer(s)[a]	Reverse primer	Probe(s)[a]
Universal	JH-F 1, JH-F all	JH-R	JH-P uni
Multiplex (group I or other)	JH-F 1, JH-F all	JH-R	JH-P 1, JH-P all
Group II	JH-F 1, JH-F all	JH-R	JH-P 2
Group XII	JH-F 1, JH-F all	JH-R	JH-P 12
Group IV			
All isolates	JH-F CSPW, JH-F LY, JH-F EAT	JH-R	JH-P uni
United States	JH-F LY	JH-R	JH-P uni
West Africa	JH-F CSPW	JH-R	JH-P uni
East Africa	JH-F EAT	JH-R	JH-P uni

[a] Where more than one F primer or probe is included, this means they should be mixed in equal amounts to provide the specificity indicated.

All DNAs were successfully amplified by both real-time PCR assays (Table 3), producing C_T values of 32 or less. The differences in the absolute C_T values between samples is most likely a reflection of differences in the titers of phytoplasma present in the plants from which the samples were obtained, since the amount of total (plant plus phytoplasma) DNA used for each assay was within the range of 0.5 to 1.0 μg. The multiplex assay was also evaluated for its capacity to identify DNA as belonging to either group 16SrI or any other group apart from 16SrI, making use of the fact that the 16SrI probe was labeled with 6-carboxyfluorescein (FAM) fluorescence while the probe for any other group was labeled with VIC (Applied Biosystems) (Table 3). Therefore, by using both probes simultaneously and determining which one generated a signal, it was possible to identify whether or not the phytoplasma belonged to group 16SrI, while the presence of no signal with either probe indicated that no phytoplasma was present. The assay was found to be 100% successful at categorizing DNA as either group 16SrI or another group, confirming the specificities of the probes to the respective DNAs (Table 3). To determine whether the competition between the two probes in the multiplex assay decreased the sensitivity of detection, the multiplex and universal assays were directly compared, with a range of DNAs tested in parallel. C_T values were found to be around 1 to 2 cycles higher in most cases with the multiplex assay than with the universal assay (Table 3), indicating a slight but insignificant decrease in sensitivity. There were also some instances where the multiplex assay resulted in C_T values lower than those with the universal assay, for example, with flavescence dorée FD-C and FD-D, indicating that the multiplex probes bind more efficiently to DNA from these groups.

Development of group-specific assays. To determine whether this region of the 23S rRNA gene (see Fig. S1 in the supplemental material) could be used to generate additional primers and probes that could be used to assign isolates to their respective 16Sr groups in specific assays, a range of primers and/or probes were designed to give specific detection of 16SrII, -IV, and -XII group isolates (Tables 1 and 2). The results are shown in Table 4. Where a specific probe was

TABLE 3. Phytoplasma isolates used in this study and real-time PCR results obtained using the multiplex and universal assays

Phytoplasma strain or bacterial source (isolate designation)	16Sr group	DNA source[a]	Average C_T value		
			Universal	Multiplex	
				16SrI probe	Other group probe
Negative control containing water			ND[c]	ND	ND
B. gladioli					
Pure culture[b]			38.5	ND	38.1
Inoculated *Allium* foliage[b]			ND	ND	ND
Inoculated *Allium* bulb[b]			ND	ND	ND
Chrysanthemum yellows (CHRYM)[b]	I-A	DNA, A. Bertaccini	21.8	21.1	ND
Rehmannia glutinosa (RG)	I-B	Plant, J. Přibylová	22.0	20.0	ND
Black currant reversion disorder (BCRD)	I-C	Plant, J. Přibylová	32.0	26.7	ND
Strawberry green petal (SGP)	I-C	Plant, J. Přibylová	20.5	19.3	ND
Cleome phyllody (CLP)	II-A	DNA, A. Bertaccini	15.9	ND	18.7
Crotalaria saltiana phyllody (FBPSA)	II-C	Plant, R. Mumford	20.4	ND	19.5
Soybean phyllody (SOYP)	II-C	Plant, P. Jones	23.1	ND	22.5
Fava bean phyllody (FBP)	II-C	Plant, P. Jones	18.4	ND	17.2
Sweet potato little leaf (SPLL)	II-D	Plant, P. Jones	17.9	ND	18.1
Vinca coconut phyllody (VCP)	II-?[d]	Plant, P. Jones	18.1	ND	19.0
Peach western X (CX)	III-A	DNA, A. Bertaccini	17.9	ND	20.6
Poinsettia branching factor (JR1)	III-H	Commercial plant, United Kingdom	21.8	ND	28.3
Coconut lethal yellowing (*Adonidia merrillii*) (LYAM)[b]	IV-A	DNA, N. Harrison	17.1	ND	18.7
Tanzanian lethal decline (TLD)	IV-B	DNA, P. Jones	24.2	ND	26.5
Ghanaian Cape St. Paul wilt (CSPWD)	IV-C	DNA, J. Nipah	23.1	ND	25.7
Elm witches' broom (ULW)[b]	V-A	DNA, A. Bertaccini	18.1	ND	19.7
Flavescence dorée C (FD-C)[b]	V-C	DNA, A. Bertaccini	30.7	ND	27.3
Flavescence dorée D (FD-D)[b]	V-D	DNA, A. Bertaccini	28.6	ND	25.7
Brinjal little leaf (BLL)	VI-A	Plant, P. Jones	19.5	ND	21.3
Catharanthus phyllody (CPS)	VI-C	DNA, A. Bertaccini	21.8	ND	22.0
Ash yellows (ASHY-1)[b]	VII-A	DNA, A. Bertaccini	18.8	ND	21.3
Pigeonpea witches' broom (PPWB)[b]	IX	DNA, N. Harrison	13.8	ND	14.6
Apple proliferation (AP-15)[b]	X-A	DNA, A. Bertaccini	19.7	ND	20.8
German stone fruit yellows (GSFY-1)[b]	X-B	DNA, A. Bertaccini	19.5	ND	20.8
Pear decline (PD)[b]	X-C	DNA, A. Bertaccini	22.7	ND	22.2
Napier grass stunt (NGS)	XI	Plant, P. Jones	23.3	ND	29.5
Stolbur of pepper (STOL)[b]	XII-A	DNA, A. Bertaccini	16.1	ND	16.9
Mexican periwinkle virescence (MPV)[b]	XIII	DNA, N. Harrison	12.7	ND	12.9
Bermuda grass white leaf (BGWL)[b]	XIV	DNA, J. Nejat	13.8	ND	16.4

[a] Samples were obtained as DNA preparations (DNA) or plant material (Plant) from the people named, and their affiliations are given in Acknowledgments.
[b] The same DNA preparation was used for the evaluations of both assays.
[c] ND, not detected, i.e., above the maximum C_T value of 40 cycles.
[d] ?, subgroup not determined.

designed for 16SrII or 16SrXII assays, positive results were generated only from isolates of the desired groups. The 16SrIV coconut lethal yellowing type phytoplasmas have generally been classified as 16SrIV-A from the Americas and the Caribbean, 16SrIV-B from Tanzania, and 16SrIV-C from Nigeria and Ghana. Therefore, a series of specific forward primers were designed to discriminate between the subgroups using a common probe. Some slight cross-amplification of other subgroups occurred, but the C_T values for these other groups were much higher (>36) than those for the specific 16SrIV isolates. To assess the binding action of each of the individual 16SrIV forward primers, these primers were evaluated separately and combined on a series of DNAs from each geographic location (Table 5). Again, the 16SrIV-B and 16SrIV-C forward primers demonstrated some non-subgroup-specific binding. However there was a substantially higher C_T value (>10) when the primer was acting nonspecifically than when it was acting specifically. The 16SrIV-A forward primer bound only to 16SrIV-A DNA. When all of the primers were combined in a single assay, group 16SrIV isolates from a wide geographic area were successfully identified.

It is important to note that, with the exception of the 16SrIV group assay, all of the developed assays utilize a combination of two forward primers, one being sequence specific for groups 16SrI, -XII, and -XIII (AS branch of phytoplasmas [19]) and the other for the remaining groups (WB branch). Testing revealed that for the most efficient amplification of all groups, the primers should be mixed at equimolar concentrations and not used individually (data not shown).

Evaluating cross-reaction with other bacteria. *Burkholderia gladioli* was selected to assess the cross-reactivity of the assays, since it showed the highest degree of sequence similarity in the

TABLE 4. Real-time PCR results for three different group-specific assays

Phytoplasma strain or bacterial source (isolate designation)	16Sr group	DNA source[a]	Avg C_T value[b]		
			Group II	Group IV	Group XII
Negative control containing water	—[c]		ND[d]	ND	ND
B. gladioli					
Pure culture	—		ND	ND	ND
Inoculated *Allium* foliage	—		ND	ND	ND
Inoculated *Allium* bulb	—		ND	ND	ND
Rehmannia glutinosa (RG)	I-B	Plant, J. Přibylová	ND	35.7	—
Black currant reversion disorder (BCRD)	I-C	Plant, J. Přibylová	ND	—	—
Strawberry green petal (SGP)	I-C	Plant, J. Přibylová	ND	38.7	ND
Fava bean phyllody (FBP)	II-C	Plant, P. Jones	19.2*	38.0	—
Crotalaria saltiana phyllody (FBPSA)	II-C	Plant, R. Mumford	19.5*	ND	—
Soybean phyllody (SOYP)	II-C	Plant, P. Jones	20.9*	ND	—
Sweet potato little leaf (SPLL)	II-D	Plant, P. Jones	26.7*	ND	ND
Sweet potato little leaf (SPLL)	II-D	Plant, P. Jones	19.3*	—	—
Vinca coconut phyllody (VCP)	II-?[e]	Plant, P. Jones	19.3*	ND	—
Peach western X (CX)	III-A	DNA, A. Bertaccini	—	ND	—
Poinsettia branching factor (JR1)	III-H	Commercial plant, United Kingdom	ND	—	ND
Coconut lethal yellowing (*Adonidia merrillii*) (LYAM)	IV-A	DNA, N. Harrison	—	20.0*	—
Coconut lethal yellowing (*Cocus nucifera*) (LYCN)	IV-A	DNA, N. Harrison	—	19.9*	—
Coconut lethal yellowing (*Hyophorbe verschaffeltii*) (LYHV)	IV-A	DNA, N. Harrison	ND	21.7*	ND
Coconut lethal yellowing (*Phoenix rupicola*) (LYPR)	IV-A	DNA, N. Harrison	—	18.9*	—
Tanzanian coconut lethal decline (Kifumangao) (TLD1)	IV-B	DNA, P. Jones	—	26.6*	—
Tanzanian coconut lethal decline (Chambezi) (TLD2)	IV-B	DNA, P. Jones	—	23.1*	—
Ghanaian Cape St. Paul wilt (Daboase) (CSPWD1)	IV-C	DNA, J. Nipah	ND	20.6*	—
Ghanaian Cape St. Paul wilt (Fasen) (CSPWD2)	IV-C	DNA, J. Nipah	—	20.8*	—
Ghanaian Cape St. Paul wilt (Dixcove) (DIXCOVE)	IV-C	DNA, P. Jones	—	18.2*	—
Nigerian coconut lethal decline (LDN)	IV-C	DNA, P. Jones	—	18.2*	—
Rubus stunt (RuS)	V	DNA, A. Bertaccini	—	37.0	—
Brinjal little leaf (BLL)	VI-A	Plant, P. Jones	ND	36.3	—
Ash yellows (ASHY-1)	VII-A	DNA, A. Bertaccini	—	36.6	—
Pigeonpea witches' broom (PPWB)	IX	DNA, N. Harrison	ND	—	—
Flower stunting (BVK)	XI	DNA, A. Bertaccini	—	ND	—
Napier grass stunt (NGS)	XI	Plant, P. Jones	ND	—	ND
Stolbur of pepper (STOL)	XII-A	DNA, A. Bertaccini	—	ND	21.3*
Cordyline phytoplasma (CPF)	XII	DNA, R. Mumford	—	—	23.8*

[a] Samples were obtained as DNA preparations (DNA) or plant material (Plant) from the people named, and their affiliations are given in Acknowledgments.
[b] *, the assay should detect these isolates.
[c] —, not tested.
[d] ND, not detected, i.e., above the maximum C_T value of 40 cycles.
[e] ?, subgroup not determined.

regions of importance relating to primer and probe binding. Cultures of *B. gladioli* pv. *alliicola* (NCPPB 3307) were acquired from the National Collection of Plant Pathogenic Bacteria (NCPPB) and grown on nutrient agar (Oxoid Ltd., Cambridge, United Kingdom). DNA extractions were performed from cultures by use of a Wizard genomic DNA purification kit (Promega Corporation, Madison, WI). The same cultures were also used to inoculate onion (*Allium cepa*); infection was allowed to establish until symptoms were evident on both the foliage and the bulb, and then samples of both tissue types were used for separate DNA extractions using the cetyl trimethyl ammonium bromide method of Doyle and Doyle (7). To confirm that *B. gladioli* infection was established, terminal restriction fragment length polymorphism was performed as described by Hodgetts et al. (12), and a terminal restriction fragment of 387 bp was found, confirming infection by *Burkholderia* spp. (as predicted using the available sequence data [results not shown]). Table 3 shows that with both the universal and the multiplex assay, there was no detection of *Burkholderia* spp. when DNA from heavily infected plants was used. There was, however, detectable amplification (C_T values of >38), close to the maximum threshold level of 40 cycles, when highly concentrated bacterial genomic DNA samples (extracted from bacteria in pure culture) were used. None of the group-specific assays resulted in detection with any of the *B. gladioli* samples (Table 4). These findings are similar to those described by Christensen et al. (4) for their universal real-time PCR assay based on the 16S rRNA gene, which also gave C_T values in the range of 17 to 30 for the phytoplasma isolates used and C_T values of 35 to 37 for pure DNA from some of the other bacteria tested, such as *Pseudomonas putida* and *Rhodococcus equi.*

To assess the specificity of the assays against a range of other bacteria typically found on plant material (present as naturally occurring concomitant bacteria or due to growth on plant material between sampling and testing), DNA extractions were performed (7) on 45 samples sent to CSL for routine bacteriological diagnostic testing; these samples represented a range

TABLE 5. Real-time PCR results for the four different group IV assays

Control or isolate designation (16Sr group)	Phytoplasma strain	Avg C_T value[a]			
		Group 16SrIV-C assay	Group 16SrIV-B assay	Group 16SrIV-A assay	Assay for all group IV isolates
Negative control		ND[b]	ND	ND	ND
CSPWD1 (16SrIV-C)	Ghanaian Cape St. Paul wilt (Daboase)	21.5*	35.2	ND	21.5*
TLD1 (16SrIV-B)	Tanzanian coconut lethal decline (Kifumangao)	35.1	25.0*	ND	25.1*
TLD2 (16SrIV-B)	Tanzanian coconut lethal decline (Chambezi)	34.2	23.1*	ND	23.1*
LYAM (16SrIV-A)	Coconut lethal yellowing (*Adonidia merrillii*)	ND	ND	19.6*	19.3*
LYPR (16SrIV-A)	Coconut lethal yellowing (*Phoenix rupicola*)	ND	ND	19.6*	19.2*
RG (16SrI-B)	*Rehmannia glutinosa*	36.7	ND	ND	35.9
FBPSA (16SrII-C)	*Crotalaria saltiana* phyllody	39.0	ND	ND	37.3
NGS (16SrXI)	Napier grass stunt	ND	ND	ND	ND

[a] *, the assay should detect these isolates.
[b] ND, not detected, i.e., above the maximum C_T value of 40 cycles.

of 10 plant species (potato, tobacco, tomato, pepper, petunia, barley, coconut, brugmansia, canna, and camellia). The COX assay (22) was used to confirm the presence of amplifiable DNA in the extracts. None of these samples gave any identifiable amplification with any of the phytoplasma-specific primers and probes, indicating that no phytoplasmas were present and that the assays did not detect any bacteria likely to be present on these samples (results not shown).

Evaluation of sensitivity. To assess the sensitivity of the assays, dilution series of two phytoplasma DNA samples from different 16Sr groups (diluted in either water or host plant DNA) were evaluated for both the universal and the multiplex assay and for the group II assay. DNA was quantified using a Nanodrop ND spectrophotometer, and 10-fold dilutions were prepared up to 1/100,000 in sterile distilled water (for isolates RG and FBP) or in healthy plant sap (for isolates RG and FBPSA) and evaluated in duplicate by real-time PCR. As anticipated, each 10-fold decrease in DNA concentration produced a drop of approximately 3 C_T, and all dilutions were detected (Table 6). The dilution series in water was also evaluated by conventional PCR, and all dilutions were detected by first-round PCR with primer pair P1 and P7, although the bands were very faint at the lowest dilutions (see Fig. S2 in the supplemental material).

In a further series of experiments to determine whether phytoplasmas could be detected more readily by the real-time PCR assay than by conventional nested PCR, two DNA extracts from plants which were known to have low titers of phytoplasma and which had previously yielded only weak nested-PCR results (see Fig. S3 in the supplemental material) were analyzed by both the universal and the multiplex assay. In the universal assay, the 16SrIII vaccinium witches' broom isolate gave a C_T value of 35.5, while in the multiplex assay it gave a C_T value of 36.8 with the probe for groups other than group I and was not detectable with the group I probe. Conversely, the 16SrI primula blue isolate gave a C_T value of 35.0 with the universal assay and a C_T value of 37.5 with the group I multiplex assay and was not detectable with the assay for groups other than group I. These results indicate a sensitivity at least equal to that of nested PCR.

Potential for further development of the assays. In this brief report, we have described a rapid phytoplasma diagnostic test with both a universal assay and a multiplex assay (which determines whether the phytoplasma present is in group 16SrI or in a group other than 16SrI) and have also shown that the same region of the 23S rRNA gene can be used to develop alternative group-specific primers and probes. There were some phytoplasma taxonomic groups and subgroups that were not tested

TABLE 6. Real-time PCR results from a dilution series in water and healthy host plant sap

Dilution series and isolate designation (16Sr group)	Isolate source	Assay	Dye	Avg C_T value				
				Neat	1/100 dilution	1/1,000 dilution	1/10,000 dilution	1/100,000 dilution
Water								
RG (16SrI-B)	*Rehmannia glutinosa*	Multiplex	FAM	20.8	27.4	30.9	35.2	38.9
	Rehmannia glutinosa	Multiplex	VIC	ND[a]	ND	ND	ND	ND
	Rehmannia glutinosa	Group II	TET[b]	ND	ND	ND	ND	ND
FBP (16SrII-C)	Fava bean phyllody	Multiplex	FAM	ND	ND	ND	ND	ND
	Fava bean phyllody	Multiplex	VIC	19.1	25.3	29.0	32.5	35.8
	Fava bean phyllody	Group II	TET	18.7	25.0	28.8	31.9	35.1
Healthy plant sap								
RG (16SrI-B)	*Rehmannia glutinosa*	Universal	FAM	20.7	26.9	30.6	33.9	37.5
FBPSA (16SrII-C)	*Crotalaria saltiana* phyllody	Universal	FAM	17.5	24.2	26.9	29.7	33.1

[a] ND, not detected, i.e., above the maximum C_T value of 40 cycles.
[b] TET, [TET] dye phosphoramidite.

because DNA was not available. However, 23S rRNA gene sequence alignments indicated that the primers and probes would work on isolates from all groups and subgroups for which 23S rRNA sequences are currently available in databases. The relevant forward primer, universal probe, and relevant multiplex probe all showed no nucleotide mismatches. The reverse primer shows a single mismatch (C, not G) 16 nucleotides from the 5′ end for some isolates, but this same mismatch occurs in some of the 16SrV, -VI, -IX, and -X isolates, which all worked in the multiplex and universal assays, so this does not affect primer binding and it can therefore be assumed that these isolates will amplify successfully.

To date, we have developed group-specific assays for 16SrII, 16SrXII, 16SrIV-A, 16SrIV-B, and 16SrIV-C phytoplasmas as proof of concept, but other group-specific assays could be developed as and when required. Primers and probes for the 16SrII and 16SrXII isolates have shown absolute specificity and do not detect isolates from any taxonomic group other than the one for which they were designed. This contrasts with primers and probes that have previously been designed based on the 16S rRNA gene, where cross-reaction has been shown to occur (5, 16). Through the development of group-specific assays, we have found that utilizing the universal assay with group-specific probes rather than group-specific primers provides the highest degree of 16Sr group specificity. However, when the sequence dictates that a group-specific primer be utilized rather than a probe, adequate differentiation between groups can be achieved, as shown for the 16SrIV isolates. By designing the amplicon to be approximately 180 bp and the location of the primers in relation to a hypervariable section of sequence, these assays also have the advantage of being suitable for direct sequencing of the quantitative PCR amplification products to allow identification of the 16Sr group of the sample. If sequencing were done with the reverse primer, there would be enough sequence variation to enable group identification of all 16Sr groups after approximately 60 bp (the region that would give poor quality sequence data due to the PCR amplicon not being cloned). This means that the assay can detect phytoplasma DNA and allow full group identification without the need to clone PCR products, making the process much faster and less costly. The desire to have group-specific amplification of a given isolate or 16Sr group of phytoplasmas has clearly been indicated by the number of published diagnostic techniques which have been designed with high specificity as the main objective. With this in mind, the assays that we have developed have the capacity to be tailored in a number of ways to allow the desired outcome to be achieved.

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