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Phytoplasma-Specific PCR Primers Based on Sequences of the 16S-23S rRNA Spacer Region

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In order to develop a diagnostic tool to identify phytoplasmas and classify them according to their phylogenetic group, we took advantage of the sequence diversity of the 16S-23S intergenic spacer regions (SRs) of phytoplasmas. Ten PCR primers were developed from the SR sequences and were shown to amplify in a group-specific fashion. For some groups of phytoplasmas, such as elm yellows, ash yellows, and pear decline, the SR primer was paired with a specific primer from within the 16S rRNA gene. Each of these primer pairs was specific for a specific phytoplasma group, and they did not produce PCR products of the correct size from any other phytoplasma group. One primer was designed to anneal within the conserved $tRNA^{Ile}$ and, when paired with a universal primer, amplified all phytoplasmas tested. None of the primers produced PCR amplification products of the correct size from healthy plant DNA. These primers can serve as effective tools for identifying particular phytoplasmas in field samples.

Phytoplasmas (11), also known as mycoplasma-like organisms, are wall-less prokaryotes that are pathogens of many plant species throughout the world (26). The ability to detect and identify phytoplasmas is necessary for accurate disease diagnosis. However, detection has been hampered by the inability to culture these prokaryotes in vitro. Therefore, alternative methods must be used to detect and characterize phytoplasmas. Although both serological and DNA hybridization methods have been used for this purpose in the past (3, 13, 15, 17), PCR has since proven to be a more versatile tool for detecting phytoplasmas in their plant and insect hosts (1, 5, 6, 20, 22, 23, 28). PCR primers have been developed from randomly cloned fragments of the phytoplasma genome (12, 28), as well as from phytoplasma-specific sequences within the 16S rRNA gene (1, 5, 20, 23). In some cases, however, the 16S rRNA sequences of related phytoplasmas are very similar, thus making it difficult or impossible to design PCR primers that could specifically identify a particular phytoplasma.

All phytoplasmas examined to date contain two rRNA operons, which appear to be identical (30). In this study, we examined the spacer region (SR) located between the 16S and 23S rRNA genes. Since there are fewer evolutionary constraints on this portion of the rRNA operon, there is generally greater variation in the SR sequence than in that of the 16S gene (2). This region has been used to detect mycoplasmas as contaminants in cell cultures (10), as well as to identify subspecies of *Clavibacter michiganensis* (21). We have sequenced numerous phytoplasma 16S-23S rRNA SRs and have found both conserved and highly variable areas within this region (14).

The objective of this study was to utilize the variation found within the SR to design PCR primers for several of the major phytoplasma groups. Here, we report that the SR PCR primers are specific for the intended group of phytoplasmas. This technique may be used as a diagnostic tool to identify phytoplasmas in a group-specific manner, which is necessary for epidemiological studies, analysis of vector relationships, and formulation of disease control strategies. The ability to detect specific groups of phytoplasmas would also benefit plant importation and quarantine agencies.

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MATERIALS AND METHODS

Phytoplasma isolates and DNA extraction. Isolates used in this study are listed in Table 1. DNA was extracted from healthy controls, from naturally-infected hosts, and from experimentally infected periwinkle (*Catharanthus roseus* (L.) G. Don) by a phytoplasma enrichment and cetyltrimethylammonium bromide extraction procedure (1). Healthy periwinkle and peach plants were grown in the greenhouse and never exposed to disease-transmitting insects. Approximately 1.5 g of fresh tissue was used for each extraction. Leaf petioles and midribs were used for DNA extraction from trees, while young symptomatic leaves were used for extraction from periwinkle.

Identification of phytoplasma-specific PCR primers and reaction conditions. The sequences of the SR primers used in this study were derived from sequences of the SRs from strains AT, BLTVA, PYLR2, SAY, WX, ESF-PCH, ULW, and ASHY and are presented in Table 2. Primers were also designed from the 16S rRNA genes of strains AT and ULW (Table 2). The sequences of the 16S rRNA primer P1 (5) and the pear decline primers fPD and rPDS (23) have been previously published. The SRs were sequenced by amplifying the entire 16S rRNA gene and the SR with primers P1 and P7 (Fig. 1; Table 2) and then directly sequencing the SR with primers P7 and P3 (Fig. 1; Table 2) with the double-stranded DNA cycle sequencing kit (Gibco BRL, Gaithersburg, Md.).

The following primer pairs were designed to specifically amplify DNA encoding rRNA (rDNA) of the respective phytoplasmas or phytoplasma groups: P1/BLTVAint, beet-leafhopper-transmitted virescence agent group; P1/WXint, western X-disease group; fB1/rULWS1, elm yellows group; fB1/rASHYS, ash yellows; fPD/rPDS, pear decline; P1/PYLRint, apple proliferation group; fAT/rAS, apple proliferation; fAT/rPRUS, European stone fruit yellows; P1/AYint, aster yellows group; and P1/Tint, all phytoplasmas. PCR was performed with 1× PCR buffer (Perkin-Elmer, Branchburg, N.J.); 50 ng of template DNA; 0.5 μM each primer; 150 μM (each) dATP, dCTP, dGTP, and dTTP; and 1 U of AmpliTaq DNA polymerase (Perkin-Elmer) in a final reaction volume of 30 μl. Amplification consisted of 30 cycles of the following steps: denaturation for 1 min at 94°C; annealing for 1 min at 48°C (P1/BLTVAint and P1/WXint), 50°C (fB1/rULWS1 and fB1/rASHYS), 52°C (fPD/rPDS), 53°C (P1/PYLRint), 55°C (fAT/rAS and fAT/rPRUS), or 56°C (P1/AYint and P1/Tint); and extension for 2 min at 72°C. Approximately 8 μl of each reaction mixture was electrophoresed in a 1% agarose gel, and the PCR products were visualized by UV transillumination after staining with ethidium bromide.

TABLE 1. Phytoplasmas used in this study

Phytoplasma	Host plant	Origin	Strain designation
Alder witches' broom	Alder	Germany	Alder
European stone fruit yellows	Almond	Germany	Almond1
European stone fruit yellows	Almond	Germany	Almond2
European stone fruit yellows	Peach	Germany	ESF-PCH
Apple proliferation	*C. roseus*	Germany	AT
Apple proliferation	*C. roseus*	Italy	AP
Apricot chlorotic leaf roll	*C. roseus*	Spain	ACLR
Ash yellows	*C. roseus*	New York	ASHY
Beet-leafhopper-transmitted virescence agent	*C. roseus*	California	BLTVA
Beet-leafhopper-transmitted virescence agent	Potato	Utah	BLTVA-POT
Brinjal little leaf	Eggplant	India	BLL
Elm yellows	Elm	Germany	ELM
Elm yellows	*C. roseus*	France	ULW
Lethal yellowing	Palm	Florida	LY
Maize bushy stunt	Corn	Florida	MBS
Peach yellow leaf roll	Peach	California	PYLR2
Pear decline	Pear	California	PD2
Pear decline	Pear	Germany	PD-I24
Pear decline	Pear	Germany	PD-I27
Pear decline	Pear	Germany	PD-308
Severe aster yellows	*C. roseus*	California	SAY
Stolbur of pepper	*C. roseus*	Serbia	STOL
Sunn hemp witches' broom	*C. roseus*	Thailand	SUNH
Vaccinium witches' broom	*C. roseus*	Germany	VAC
Walnut witches' broom	Walnut	Georgia	WWB
Western X-disease	Celery	California	WX

Nucleotide sequence accession numbers. The sequences obtained in this study were assigned GenBank accession numbers U54985 through U54992.

RESULTS

Phytoplasma strain-specific PCR primers were generated by using 16S-23S rRNA SR sequences (Table 2). The relative locations of the primer sequences in the SRs are depicted in Fig. 1. Each group-specific primer pair was tested for its ability to amplify an rDNA product of the correct size exclusively from DNA of diseased plants (Table 3). The expected size of the PCR amplification product generated with each of the primer pairs is shown in Table 4.

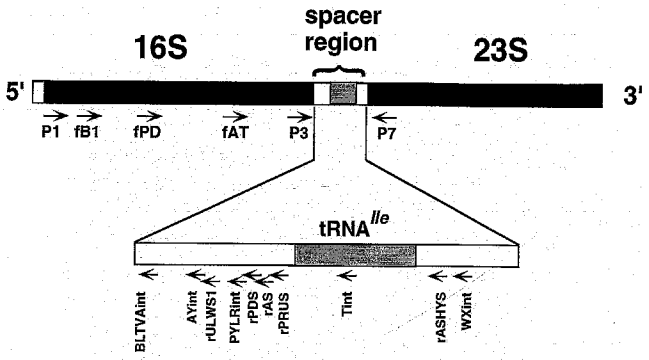

FIG. 1. Diagrammatic representation of a phytoplasma rRNA operon, including the 16S and 23S rRNA genes and the intergenic SR. The positions of oligonucleotide primers used in PCR analysis are represented as arrows. Solid or hatched bars represent coding regions.

Universal phytoplasma detection with tRNA^{Ile} PCR primer. A primer which amplified a product from all phytoplasma-infected plants was identified in a portion of the tRNA^{Ile} region within the SR (Fig. 2). A secondary PCR product, of approximately 200 bp, was amplified from all samples tested, including healthy-plant DNA samples from more than 20 plant species, with primer pair P1/Tint (Fig. 2). This band is consistently observed in the original ethidium bromide-stained gels but is frequently too faint to be reproduced in photographs of those gels.

Fruit tree phytoplasma-specific primers. Four pairs of PCR primers were generated to identify phytoplasmas in the apple proliferation group, which includes German pear decline, European stone fruit yellows, apple proliferation, California pear decline, and peach yellow leaf roll. The most specific of these primer pairs was fPD/rPDS, which amplified a PCR product of the expected size only from German pear decline, California pear decline, and peach yellow leaf roll (Fig. 3A). The primer pair generated from the European stone fruit yellows sequence (fAT/rPRUS) amplified a product of the expected size from all phytoplasmas in the apple proliferation group except the apple

TABLE 2. Sequences of the oligonucleotide primers used for PCR amplification and sequencing[a]

Primer	Location[b]	Phytoplasma source	GenBank accession no.	Oligonucleotide sequence
P1 (5)	16S	—[c]	—[c]	5′AAGAGTTTGATCCTGGCTCAGGATT3′
P3 (31)	16S	—[c]	—[c]	5′GGATGGATCACCTCCTT3′
P7 (31)	23S	SAY	M86340 (16)	5′CGTCCTTCATCGGCTCTT3′
fAT	16S	AT	X68375 (32)	5′CATCATTTAGTTGGGCACTT3′
fPD (23)	16S	PD-308	X76425 (32)	5′GACCCGTAAGGTATGCTGA3′
fB1	16S	ULW	X68376 (32)	5′GACCCTTCAAAAGGTCTTAG3′
BLTVAint	SR	BLTVA	U54987	5′GATGATTTTAGTATATATAGTCC3′
PYLRint	SR	PYLR2	U54990	5′CCCGGCCATTATTAATTTTTATC3′
AYint	SR	SAY	M86340 (16)	5′TACAATTTGCAAGCAAGTTAC3′
WXint	SR	WX	U54992	5′GACAGTGCTTATAACTTTTA3′
rAS	SR	AT	U54985	5′GGCCCCGGACCATTATTTATT3′
rPRUS	SR	ESF-PCH	U54988	5′GGCCCAAGCCATTATTGATT3′
rPDS (23)	SR	PD-308	U54989	5′CCCGGCCATTATTAATTTTA3′
rULWS1	SR	ULW	U54991	5′CGTCTTTTATATAAGAGAAACA3′
rASHYS	SR	ASHY	U54986	5′GCAGGACCGTTTATATTAATC3′
Tint	SR	—[c]	—[c]	5′TCAGGCGTGTGCTCTAACCAGC3′

[a] Numbers in parentheses are reference numbers.
[b] Location of primer within the rRNA operon, i.e., within either the 16S rRNA gene, the 235 rRNA gene, or the SR.
[c] —, primer was generated from a consensus sequence of several phytoplasmas.

TABLE 3. Results of PCR amplification from each template DNA with each primer pair

Source of template DNA[a]	Amplification by primer pair[b]: fAT/rAS	fPD/rPDS	fAT/rPRUS	P1/PYLRint	P1/WXint	fB1/rASHYS	fB1/rULWS1	P1/BLTVAint	P1/AYint	P1/Tint
AP	+			+						+
AT	+			+						+
PD-I24	+	+	+	+						+
PD-I27	+	+	+	+						+
PD-308	+	+	+	+						+
PYLR2	+	+	+	+						+
PD2	+	+	+	+						+
Almond1			+	+						+
Almond2			+	+						+
ESF-PCH			+	+						+
WX					+					+
WWB					+					+
VAC					+					+
ASHY						+				+
ULW							+			+
ELM							+			+
Alder							+			+
BLTVA								+		+
BLTVA-POT								+		+
BLL								+		+
SAY									+	+
MBS									+	+
ACLR										+
SUNH										+
STOL										+
LY										+
Healthy *C. roseus*										+
Healthy peach										+

[a] Strain designations are as in Table 1.
[b] +, a PCR product of the expected size was amplified from the template DNA.

proliferation phytoplasma (Fig. 3B). Conversely, the primer pair generated from the apple proliferation sequence (fAT/rAS) yielded products of the predicted size from all apple proliferation group isolates except European stone fruit yellows (Fig. 3C). The primer pair P1/PYLRint amplified rDNA from all phytoplasmas in the apple proliferation group (Table 3). These four primer pairs were specific for the apple proliferation group of phytoplasmas because they did not generate a PCR product of the expected size from any other test sample.

In addition to the apple proliferation group of phytoplasmas, members of the western X-disease group also infect fruit and nut trees. Isolates of western X-disease, walnut witches' broom, and vaccinium witches' broom yielded a product of the expected size only when amplified with primer pair P1/WXint (Fig. 4).

Shade tree phytoplasma-specific primers. The ash yellows and elm yellows phytoplasmas cause significant damage to ash and elm trees in the eastern U.S. and Europe (24, 25, 33). Primer pairs which could distinguish pathogens associated with each of these diseases were developed. The primer pair fB1/rASHYS specifically detected the ash yellows phytoplasma (Fig. 5A), while fB1/rULWS1 detected all members of the elm yellows group tested, including elm yellows isolated from elm

TABLE 4. Expected size of phytoplasma-specific PCR products generated from SR and 16S rRNA primer pairs

PCR primer pair	Approximate product size (bp)
fAT/rAS	500
fPD/rPDS	1,400
fAT/rPRUS	500
P1/PYLRint	1,550
P1/WXint	1,600
fB1/rASHYS	1,500
fB1/rULWS1	1,500
P1/BLTVAint	1,450
P1/AYint	1,500
P1/Tint	1,600

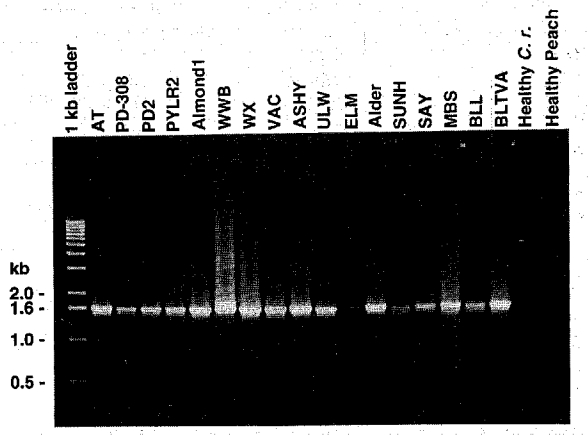

FIG. 2. Ethidium bromide-stained gel of PCR amplification products obtained by using primer pair P1/Tint. The source of target DNA is given above each lane. Strain designations are as listed in Table 1. *C. r.*, *C. roseus*.

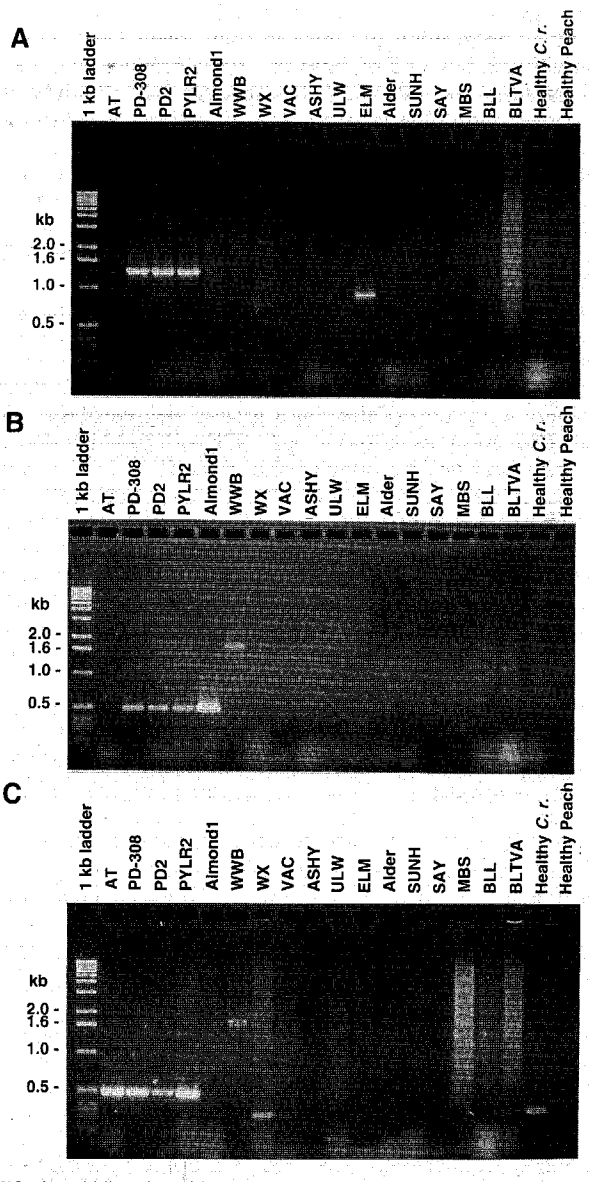

FIG. 3. Ethidium bromide-stained gels of PCR amplification products obtained by using pear decline group-specific primer pairs fPD/rPDS (A), fAT/rPRUS (B), and fAT/rAS (C). The source of target DNA is given above each lane. Strain designations are as listed in Table 1. *C. r.*, *C. roseus*.

and *C. roseus*, as well as alder witches' broom (Fig. 5B). Minor bands were amplified from samples other than those from members of the elm yellows group with primer pair fB1/rULWS1, but the products were not of the size predicted for a phytoplasma product.

Virescence- and phyllody-inducing phytoplasma-specific primers. The primer pair P1/AYint specifically detected severe aster yellows and maize bushy stunt, which are both members of the aster yellows group (Fig. 6A), but did not detect strain ACLR, a more distantly related member of the group (Table 3). Additionally, this primer pair did not detect the stolbur phytoplasma, which is related to, but genetically distinct from, the aster yellows group (Table 3).

Finally, primer pair P1/BLTVAint specifically detected members of the beet-leafhopper-transmitted virescence agent group (Fig. 6B and Table 3). A previous study indicated that

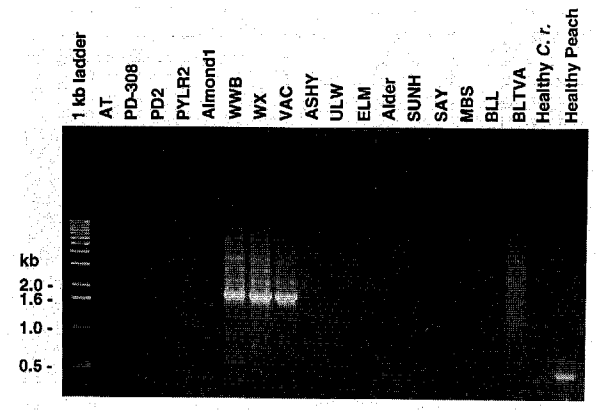

FIG. 4. Ethidium bromide-stained gel of PCR amplification products from the western X-disease group-specific primer pair (P1/WXint). The source of target DNA is given above each lane. Strain designations are as listed in Table 1. *C. r.*, *C. roseus*.

the Brinjal little leaf phytoplasma was related to the ash yellows phytoplasma on the basis of 16S rRNA sequences (29). However, the P1/BLTVAint primer pair amplified rDNA of the Brinjal little leaf phytoplasma, indicating that this pathogen is closely related to strain BLTVA. The SR sequence of strain BLL was determined later and was found to be almost identical to that of strain BLTVA, thus confirming the group identity of Brinjal little leaf and the utility of this primer pair. As was the case with all primer pairs tested, no products of the

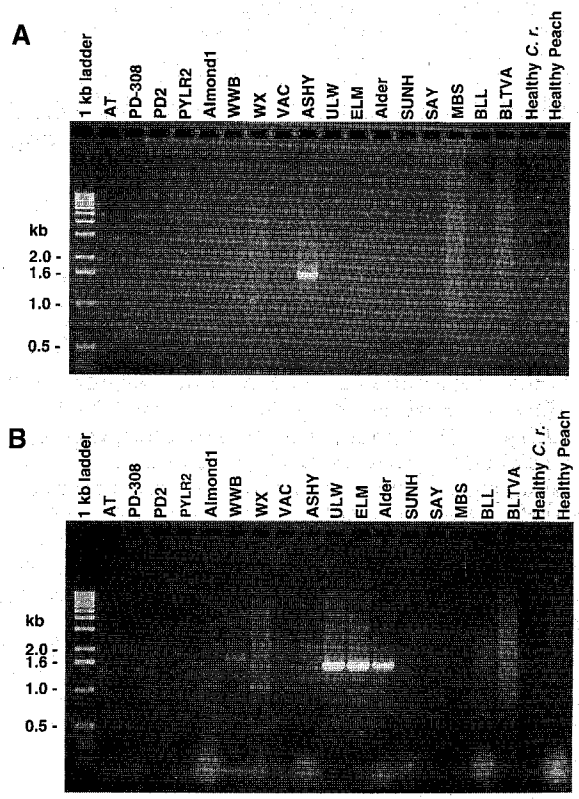

FIG. 5. Ethidium bromide-stained gels of PCR amplification products from shade tree phytoplasma-specific primer pairs fB1/rASHYS (A) and fB1/rULWS1 (B). The source of target DNA is given above each lane. Strain designations are as listed in Table 1. *C. r.*, *C. roseus*.

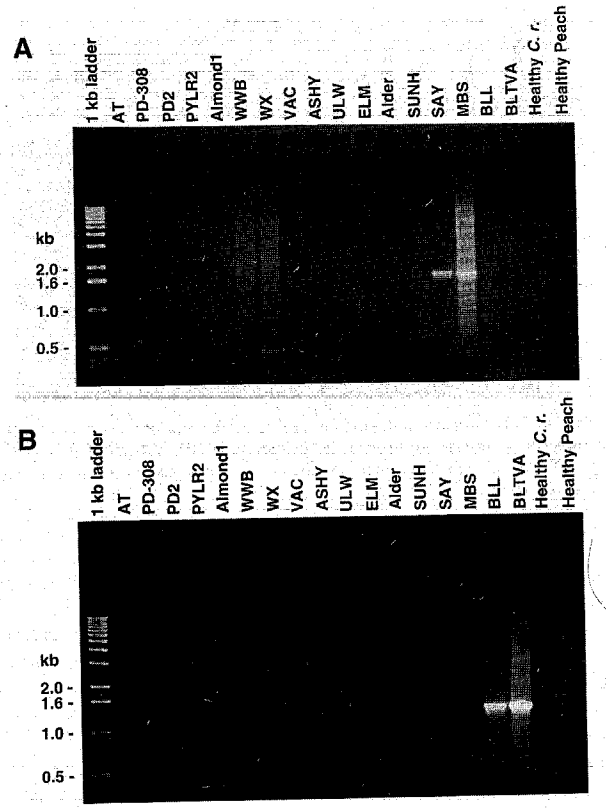

FIG. 6. Ethidium bromide-stained gels of PCR amplification products from virescence-inducing phytoplasma-specific primer pairs P1/SAYint (A) and P1/BLTVAint (B). The source of target DNA is given above each lane. Strain designations are as listed in Table 1. *C. r.*, *C. roseus*.

expected size were amplified from phytoplasmas other than those of the specific group for which the primers were designed (Table 3).

DISCUSSION

The intergenic SR was shown to be a useful region for the development of group-specific phytoplasma PCR primers. The PCR primer pair P1/Tint provides an additional tool for reliable detection of a phytoplasma infection. This primer pair produced a PCR product from all phytoplasma groups tested, regardless of the plant host from which the DNA was extracted. The P1/Tint primer pair was also tested against *Spiroplasma citri* and *Spiroplasma kunkelii*; however, no PCR product was amplified. After a sample is found to be infected with a phytoplasma with general PCR primers such as P1/Tint, it is possible to determine the group to which this organism belongs by using the more specific primers that we have developed from SR sequences. We extracted DNA from 12 different plant hosts which were known to have various titers of phytoplasmas. Using primer pair P1/Tint, we demonstrated that a PCR product of about 1.6 kb could be amplified from all of our DNA preparations. Primer pair P1/Tint also amplified a small PCR product from all plants tested, including both infected and healthy plants. This product can serve as a convenient marker that shows the absence of PCR inhibitors in the DNA template. We then used the same DNA preparations to perform the group-specific amplifications. This approach enabled us to discount the role of low phytoplasma titer or inhibitory contaminants when interpreting patterns of amplification resulting from the use of specific primers. We did occasionally see nonspecific amplification products in some plant DNA preparations (Fig. 2, 3, 5, and 6); however, these products were not of the expected size (Table 4). Nonspecific PCR products were most common in DNA extracted from walnut (Fig. 3B and C and 5B).

The primers we designed to detect phytoplasmas in the apple proliferation group, which includes German pear decline, European stone fruit yellows, apple proliferation, California pear decline, and peach yellow leaf roll, were not fully effective at distinguishing individual members within the group. The sequences of the SRs of the members of this group are not identical, so we attempted to design primers which were specific for each organism within the group. While the P1/PYLRint primer pair detected all members of the apple proliferation group (Table 1), a greater degree of specificity was obtained by using primer pair fPD/rPDS, which detected only the pear decline samples and strain PYLR2. This result was expected because the pear decline and peach yellow leaf roll phytoplasmas have identical SR sequences. However, we were not able to develop a primer pair that could detect only apple proliferation or European stone fruit yellows. In each of these cases, we amplified a PCR product from both AP and PD samples or from both PD and ESFY. Thus, while we were able to distinguish AP from ESFY, we could not distinguish either of them from PD.

SR primers were also able to differentiate between some members of the aster yellows group. The primer pair P1/AYint detected both SAY and MBS, the SR sequences of which are nearly identical (14, 31). Previous studies of 16S rRNA sequences have shown that strain ACLR is a more distantly related member of this group, while strain STOL is either a distantly related member of the aster yellows group or a member of a genetically distinct group that is closely related to the aster yellows group (9, 32). Our aster yellows group-specific primers did not detect either ACLR or STOL, indicating that these organisms are not closely related to SAY and MBS, as has been previously shown (9, 32).

Phylogenetic analysis of the SR sequences indicates that BLTVA falls in a group with ASHY (31), while analysis of the 16S sequences of BLL and ASHY places them together in a group (29). Even though BLL and BLTVA have both been shown to be related to ASHY, the primer pair fB1/rASHYS amplified products only from ASHY and not from BLTVA or BLL (Fig. 5A). Conversely, the primer pair P1/BLTVA amplified products from BLTVA and BLL, but not from ASHY (Fig. 6B). On the basis of this information, we conclude that BLTVA and BLL are more closely related to each other than to ASHY.

The PCR primers and protocols described here allow the detection of a broad range of phytoplasmas (P1/Tint) or of specific, phylogenetically defined phytoplasma groups which were previously identified (4, 9, 14, 31, 32). Some of these pathogens, such as the apple proliferation phytoplasma, are currently restricted to specific geographical areas. Governmental plant importation facilities currently expend considerable effort in testing imported plant materials for the presence of nonindigenous phytoplasmas. Given the availability of rapid, sensitive, and specific PCR assays to detect these pathogens, it is likely that PCR testing of imported germ plasm will augment or perhaps even replace the traditional graft-indexing procedures that have been used to detect woody-plant phytoplasmas. We have found that detection of woody-plant phytoplasmas by PCR is as reliable as graft indexing (8). PCR protocols, using either group-specific SR or 16S rRNA (4, 23, 27) PCR primers and employing a single set (20 to 35 cycles) of amplifications,

have resulted in reliable detection of specific phytoplasmas. The biological significance of other, low-titer, "cryptic" phytoplasmas (18, 19) that are apparently detected only by using nested PCR (70 amplification cycles) for plant materials in which only one phytoplasma genotype is detected by a single set of PCR amplifications remains unclear. The impact on plant health and the biological significance of low-titer, cryptic phytoplasmas need to be determined if PCR is to be reliably used by plant introduction regulatory agencies to detect plant pathogenic phytoplasmas.

While many PCR assays have been developed to identify phytoplasmas (5, 7, 12, 20, 23, 28), we believe that the SR primers will be exceptionally useful for three reasons. First, the majority of the SR primers can be used in conjunction with primer P1 (5), so the total number of primers necessary for group-specific phytoplasma identification is reduced. Second, because the SR region is more variable than the 16S rRNA gene, it will be comparatively easier to identify SR sequences from which primers can be designed for the detection of other phytoplasma groups not analyzed in this study. Finally, because the SR is much shorter than the full-length 16S rRNA gene, it is easier to sequence. A database of more than 60 phytoplasma SR sequences now exists (14), which should facilitate the identification of other phytoplasma clade-specific PCR primers.

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