

Development of a Real-Time, Quantitative PCR for Detection of the Columbia Basin Potato Purple Top Phytoplasma in Plants and Beet Leafhoppers

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ABSTRACT

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A quantitative, real-time "TaqMan" polymerase chain reaction assay (real-time PCR) was developed which was capable of detecting and quantifying a group 16SrVI phytoplasma in DNA extracts prepared from infected tomatoes, potatoes, and beet leafhoppers (*Circulifer tenellus*). Primers and probe were designed from the 16S rRNA gene of the Columbia Basin potato purple top phytoplasma, which is closely related to the beet leafhopper transmitted virescence agent. The detection limit in phytoplasma-infected tomato DNA was approximately 50 pg. The concentration of phytoplasma varied considerably among potato plants showing symptoms of purple top. The pathogen was readily detected in extracts from single or groups of five beet leafhoppers. As with infected potatoes, the concentration of phytoplasma in individual leafhoppers was variable. The assay also detected aster yellows (group 16SrI) and pigeon pea witches'-broom (group 16SrIX) phytoplasmas in infected periwinkle plants. The real-time PCR was at least as sensitive as the commonly used and more labor-intensive nested PCR for detection of the pathogen.

Additional keywords: insect vectors

Phytoplasmas, previously called mycoplasma-like organisms, (6,38) are wall-less prokaryotes of the class *Mollicutes*. These organisms are the causal agents of numerous plant diseases including aster yellows (AY), western X-disease, alfalfa witches'-broom, potato witches'-broom, apple proliferation, pigeon pea witches'-broom (PPWB), clover proliferation (CP), and many others (12,21,23,29). The phytoplasmas are transmitted by several species of insects, particularly leafhoppers (Cicadellidae) and psyllids (Psyllidae) (6). The aster leafhopper (*Macrostelus fascifrons* Stål) transmits the aster yellows phytoplasma, which has a broad host range (29). Another common insect, the beet leafhopper (*Circulifer tenellus* Baker), transmits the vinca virescence or beet leafhopper-transmitted virescence agent (BLTVA) (14,35). Numerous other vector species of different phytoplasmas have been identified (29).

Previous research has shown that BLTVA infects a number of agriculturally important plants (14). This organism was

identified as an important pathogen of radish seed crops in Washington (39) and Idaho (40), tomatoes in California (41), and potatoes in Utah (43). Recently, BLTVA has been implicated as the causal agent of the potato purple top and dry bean phyllody diseases in the Columbia Basin region of Washington and Oregon (25,26). The Columbia Basin potato purple top phytoplasma, which is closely related to or synonymous with BLTVA, has been frequently detected within the beet leafhopper and occasionally within *Ceratagallia* spp. leafhoppers, but has not been detected in numerous insects of other cicadellid and delphacid taxa in Washington and Oregon (9). Recently, the purple top phytoplasma has been transmitted by the beet leafhopper from radishes to potatoes in greenhouse trials (J. E. Munyaneza and J. M. Crosslin, *unpublished*).

The phytoplasmas, in contrast to Spiroplasma, have not been successfully cultured *in vitro* (6,29). Therefore, other methods have been devised for their study. These include serology (8,11), use of labeled DNA hybridization probes (10,13,27,28), and more recently the polymerase chain reaction (PCR) (5,12,30,31,42). Gundersen and Lee (16) described a more sensitive nested PCR procedure, in which a portion of the first round reaction was removed, diluted, and used in a second PCR with primers internal to the first pair.

The advent of PCR and rapid cloning and sequencing technologies has allowed accumulation of genomic sequence infor-

mation on the phytoplasmas, particularly the 16S rRNA genes and the 16S-23S intergenic spacer region. This information has led to phylogenetic separation of the phytoplasmas into groups, designated by 16Sr and Roman numerals from I to XIV. For example, the aster yellows group is 16SrI and the clover proliferation group (18), which includes BLTVA, comprises group 16SrVI (30).

The most recent innovation in the detection of phytoplasmas is the real-time, quantitative PCR (real-time PCR) methodology (2,7,20,33). The real-time PCR can be completed more quickly than the nested PCR procedures. Real-time PCR reduces the chances of contamination since the reaction vessels do not need to be opened between the first round and nested reactions, and it also eliminates the need to analyze the reaction products by gel electrophoresis. The goal of the research described herein was the development of a real-time PCR for group 16SrVI phytoplasma in potatoes, tomatoes, and beet leafhopper vectors that would eliminate the need to use nested PCR.

MATERIALS AND METHODS

Plant materials and insects. Potatoes (*Solanum tuberosum* L.) showing symptoms of purple top disease were obtained from commercial potato fields and research plots in the Columbia Basin region of southern Washington and northern Oregon in middle to late summer. Most potato samples were cultivars Ranger Russet or Russet Burbank. Symptomatic shoots of selected plants were tip-grafted onto tomatoes (*Lycopersicon esculentum* Mill.) in the greenhouse. Symptoms on field-grown potato included stunting, leaf rolling, chlorosis, and purple discoloration of the leaflets. Some infected potatoes produced aerial tubers. The foliar symptoms on infected tomatoes consisted of shortened internodes, bunched growth, purple discolorations, and reduced flowering typical of "big bud" symptoms (41). Periwinkle (*Catharanthus roseus* (L.) G. Don) tissues infected with aster yellows and pigeon pea witches'-broom phytoplasmas were obtained from N. Harrison, University of Florida, Fort Lauderdale, and grafted onto healthy periwinkle plants maintained in a greenhouse. Symptoms included flower virescence, stunting, chlorosis, and motting of leaves. Insects in and near potato fields were collected with sweep nets and

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identified at the Yakima Agricultural Research Laboratory (9). Insects identified as beet leafhoppers were stored in 70% ethanol at room temperature until used for nucleic acid extraction.

Nucleic acid extractions from plants and insects. Midveins, petioles, and small stem pieces of potato, tomato, and periwinkle were excised (approximately 200 to 500 mg per sample) with a new razor blade and placed into a mesh grinding bag (Agdia, Inc., Elkhart, IN). Total nucleic acid was extracted using the method of Presting et al. (36), with minor modifications. The tissue was triturated with 1.2 ml of buffer (100 mM Tris-HCl, pH 8.0, 500 mM NaCl, 50 mM disodium ethylenediamine tetraacetic acid, 10 mM 2-mercaptoethanol) using a large pestle. Six hundred microliters of the solution was placed in a microcentrifuge tube containing 60 μ l of 10% sodium dodecyl sulfate, mixed, and incubated 10 min at 65°C. Two hundred microliters of acidified 5 M potassium acetate (37) was then added, mixed by vortexing, and the solution incubated on ice 10 min. Debris was pelleted by centrifugation at 14,000 \times g for 10 min, and 600 μ l of the clarified supernatant was transferred to a new microcentrifuge tube. After addition of 300 μ l of isopropanol, the solution was mixed, held on ice 10 min, and centrifuged for 10 min. The nucleic acid pellet was washed once

with 70% ethanol, air-dried, and resuspended in 300 μ l of sterile distilled water.

Nucleic acids were extracted from insects using the CTAB extraction method of Zhang et al. (45) but without grinding in liquid nitrogen. Briefly, the insects (individually or in groups of five) were washed once with CTAB buffer, then ground in 600 μ l of fresh buffer using a micropestle, and processed as described. Nucleic acids were resuspended in 50 μ l of sterile distilled water for single insect extractions or in 100 μ l for the five insect extractions. In some tests, insects were extracted using the procedure described above for plant tissue, except the insects were ground in a 1.5-ml microcentrifuge tube with a micropestle. Additional extracts were prepared from plants or insects using the Fast-DNA procedure as described by the manufacturer (Bio 101, Inc., Carlsbad, CA).

The DNA or total nucleic acid extracts were quantified using the double-stranded DNA specific reagent Picogreen (Invitrogen, Carlsbad, CA) and a fluorometer (Turner Designs, Inc., Sunnyvale, CA) as recommended by the manufacturers. The DNA concentrations of stock solutions were adjusted to 5 to 50 ng/ μ l. Tenfold dilution series of some quantified DNA extracts were prepared in sterile distilled water for use in determining dilution endpoints and preparation of standard curves.

Design of real-time PCR primers and probes. The DNA sequence of a portion of the 16S rRNA gene of the Columbia Basin potato purple top phytoplasma (GenBank accession AY692280; 9) was evaluated using the Primer Express software (Applied Biosystems, Inc. [ABI], Foster City, CA). Candidate primer and probe sequences were tested for homology to phytoplasma and other prokaryotic sequences using BLAST (1). Primers 16Sp303F, 16Sp378R, and TaqMan probe 16TM329 (Table 1) were selected because they showed homology to several phytoplasma sequences and little homology with other prokaryotic 16S rRNA genes. Primers 16Sr303F and 16Sr378R amplify a 75-bp segment corresponding to nucleotides 1611 to 1686 of AY692280. The probe was synthesized and labeled with 6-carboxyfluorescein (6-FAM) and tetra-methylcarboxyrhodamine (TAMRA) by ABI. Primers used for standard PCR reactions and real-time PCR (Table 1) were obtained from Sigma-Genosys (The Woodlands, TX).

PCR and real-time PCR. First-round reactions of nested PCR (16) utilized phytoplasma universal primers P1 and P7 (Table 1). Reaction mixtures contained 5 μ l of 10 \times PCR buffer (Promega, Madison, WI), 0.5 μ l of 10 mM (each) dNTP mixture, 1 μ l each 20 μ M primer solution, 37.3 μ l of sterile distilled water, and 0.2 μ l (1 U) of *Taq* polymerase (Promega). Five microliters of DNA extracts varying in concentration were added; the reactions were overlaid with mineral oil and incubated 2 min at 94°C, then 30 cycles of 94°C for 15 s, 55°C for 90 s, 72°C for 90 s, followed by a final extension of 5 min at 72°C, then held at 4°C. For the nested reaction, 5 μ l of the P1/P7 reaction was removed, diluted with 95 μ l of sterile distilled water, and 2 μ l was used in reactions as above, except 5 μ l of Rediload (Invitrogen) and primers fU5/BLTVA-int (Table 1) were included in the reaction mix and the amount of water was adjusted as necessary. The nested primer pair fU5/BLTVA-int specifically amplifies group 16SrVI phytoplasmas (9,42). For phytoplasma group-nonspecific amplification, the nested primers were fU5/rU3 (34). Amplification conditions were as described above. Ten microliters of the reactions were subjected to electrophoresis in 1.5% agarose gels, stained with ethidium bromide, and visualized with UV light (37). The fU5/BLTVA-int and fU5/rU3 amplicons are approximately 1,200 and 880 bp, respectively.

Real-time PCR reactions were performed in Optical Tubes (ABI). The 50- μ l reactions consisted of 25 μ l of 2 \times TaqMan Universal PCR master mix (ABI), 900 nM each primer (16Sp303F and 16Sp378R), 250 nM probe 16TM329, sterile distilled water to 45 μ l, and 5 μ l of the DNA sample. Most test reactions contained 25 ng of DNA and were tested in duplicate or triplicate. Because of the low yield of DNA from some insect samples, these were

Table 1. Oligonucleotide primers and TaqMan probe sequences used for nested and real-time quantitative polymerase chain reaction (PCR) of phytoplasma in plants and insects

Primer or probe	Sequence (5' to 3')	Reference
16Sp303F	agggcctatagctcagttggtaga	This paper
16Sp378R	gtggcctaataaggactgaac	This paper
Probe 16TM329	6Fam-cacacgcctgataagcgtgaggtcg-Tamra	This paper
P1	aagagtttgatcctggctcaggatt	13
P7	cgtcctcatcggctctt	38
fU5	cggcaatggaggaaact	34
rU3	ttcagctaccctttgtaaca	34
BLTVA-int	gatgatttagtatatagtc	42

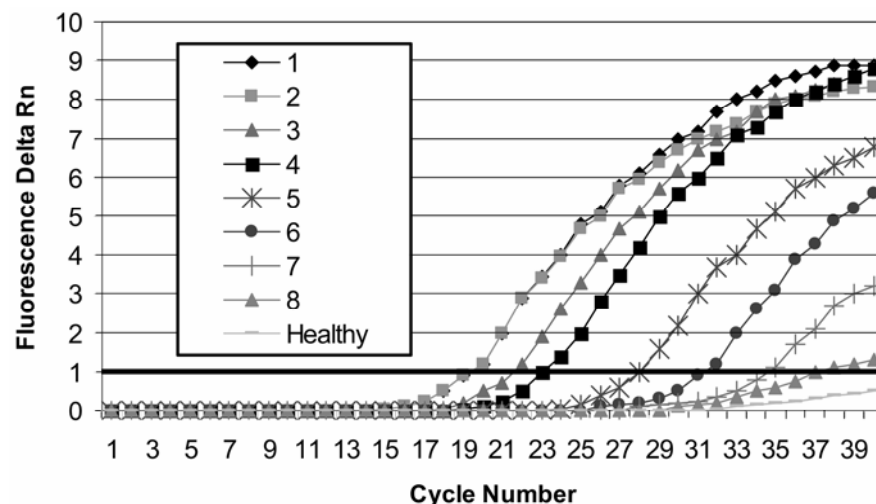


Fig. 1. Real-time polymerase chain reaction (PCR) on 25 ng of DNA from foliage of eight potato plants showing symptoms of purple top that were positive for phytoplasma by nested PCR, and a healthy 'Shepody' potato ($R^2 = 0.994$, slope -3.65 , threshold 1.0).

often tested singly. DNA samples were diluted 10-fold in water and tested in triplicate for development of standard curves using BLTVA-infected tomato DNA. Real-time PCR was conducted on an ABI 7000 instrument with the default parameters (50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min).

Data analysis. After real-time PCR reactions were completed, threshold values were adjusted to 1.0 to enable comparison of data sets. Standard curves were computed using

triplicate wells of BLTVA-infected tomato DNA in 10-fold dilutions from 500 ng to 0.005 ng per well. The mean threshold cycle (Ct) values were calculated as were standard deviation among the replicates. Quantity calculations in potato and insect samples were based upon the infected tomato DNA standard curve.

RESULTS AND DISCUSSION

The real-time PCR assay reliably detected the group 16SrVI phytoplasma in

DNA from infected tomatoes, potatoes, and the aster yellows (group 16SrI) and potato witches'-broom (group 16SrIX) phytoplasma-infected periwinkles (Table 2). The dilution endpoint in DNA from infected tomatoes was 5 to 50 pg by real-time PCR, and nested PCR detected phytoplasma in 500 pg but not 50 pg of DNA from the infected tomato (Table 2). A dilution series of 5 pg to 50 ng of DNA from six potato plants showing symptoms of purple top indicated that there was consid-

Table 2. Real-time polymerase chain reaction (PCR) and nested PCR results on healthy and beet leafhopper-transmitted virescence agent (BLTVA)-infected tomato, aster yellows (AY), and pigeon pea witches'-broom (PPWB)-infected periwinkles, and six potato plants showing symptoms of potato purple top (R² 0.9988, slope -3.42, intercept 31.67, threshold 1.0)^a

Sample	DNA concentration	Replications	Std. Dev.	Ct ^b	Quantity ^c	Nested PCR ^d
BLTVA-infected tomato	500 ng	3	0.037	22.53	500	+
	50 ng	3	0.004	25.85	50	+
	5 ng	3	0.057	29.11	5	+
	500 pg	3	0.035	32.60	0.5	+
	50 pg	3	0.089	36.47	0.05	-
	5 pg	3	0.151	39.40	0.005	-
Healthy tomato	50 ng	3	0.000	-- ^e	--	-
AY-infected periwinkle	50 ng	1	--	19.74	3068.70	- ^f
	5 ng	1	--	23.00	343.02	-
	500 pg	1	--	26.17	40.48	-
PPWB-infected periwinkle	50 ng	1	--	30.98	1.59	-
	5 ng	1	--	34.35	0.17	-
	500 pg	1	--	37.03	0.03	-
Healthy periwinkle	50 ng	1	--	--	--	-
	5 ng	1	--	--	--	-
	500 pg	1	--	--	--	-
Symptomatic potato A	50 ng	2	0.058	31.16	1.45	+
	5 ng	2	0.139	34.30	0.17	+
	500 pg	2	0.302	37.62	0.02	-
	50 pg	2	--	--	--	-
	5 pg	2	--	--	--	-
Symptomatic potato B	50 ng	2	0.495	35.75	0.66	+
	5 ng	2	0.251	38.00	0.14	+
	500 pg	2	--	--	--	-
	50 pg	2	--	--	--	-
	5 pg	2	--	--	--	-
Symptomatic potato C	50 ng	2	0.000	22.55	463.95	+
	5 ng	2	0.047	26.03	44.43	+
	500 pg	2	0.067	29.56	4.15	+
	50 pg	2	0.021	33.00	0.41	+
	5 pg	2	0.089	36.36	0.04	-
Symptomatic potato D	50 ng	2	0.054	21.24	1122.22	+
	5 ng	2	0.102	24.55	121.11	+
	500 pg	2	0.006	28.07	11.33	+
	50 pg	2	0.053	31.63	1.02	+
	5 pg	2	0.039	35.08	0.10	+
Symptomatic potato E	50 ng	2	0.010	34.10	0.20	+
	5 ng	2	0.175	37.20	0.02	+
	500 pg	2	--	--	--	-
	50 pg	2	--	--	--	-
	5 pg	2	--	--	--	-
Symptomatic potato G	50 ng	2	0.025	19.25	4280.44	+
	5 ng	2	0.022	22.73	409.99	+
	500 pg	2	0.145	25.94	47.51	+
	50 pg	2	0.016	29.40	4.60	+
	5 pg	2	0.009	33.10	0.38	-

^a Real-time PCR used primers 16Sp303F and 16Sp378R and probe 16TM329.

^b Threshold cycle.

^c Quantity is expressed as ng of phytoplasma-infected tomato DNA.

^d Primers were P1/P7//fU5/BLTVA-int. (+) and (-) indicate that the expected band was present or absent, respectively, after agarose gel electrophoresis.

^e Not detected.

^f AY and PPWB phytoplasmas are not amplified with the group 16SrVI-specific nested PCR primers, fU5/BLTVA-int.

erable variability in the amount of phytoplasma DNA in these plants (Table 2). This result was confirmed when 25 ng of DNA from eight purple top-symptomatic plants were tested and the threshold cycle values (Ct) were plotted (Fig. 1). The data illustrated in Figure 1 and Table 2 correlate to a several thousand-fold difference in phyto-

plasma DNA concentration among the extracts from the purple top-symptomatic potato plants.

The phytoplasma was also detected by real-time PCR in individual and five-insect groups of beet leafhoppers (Table 3). The quantity of phytoplasma DNA, expressed as nanograms of infected tomato DNA,

also varied among the insect extracts but not as dramatically as DNA from the symptomatic potato plants. Detection of phytoplasma DNA in beet leafhoppers by real-time PCR and nested PCR results on the same samples were nearly always in agreement. In only one case did a sample (single leafhopper sample 14) give a positive nested PCR result and fail to produce a real-time PCR signal. Generally, real-time PCR quantity estimates of about 0.1 ng or greater produced a positive nested PCR result (Tables 2 and 3).

Many researchers have reported the use of nested PCR for detection of phytoplasmas (16,19,22,32). Although nested PCR may increase sensitivity, it also greatly increases the chance for contamination, leading to false-positive results, since the first-round reaction vessel must be opened, an aliquot removed, diluted, and used in the second reaction. Reaction components, the number of tubes necessary, and labor are also increased in the nested PCR procedure compared with the real-time PCR method. A third advantage of the real-time PCR method over nested PCR is elimination of the need for gel electrophoresis of the PCR products.

To our knowledge, this is the first report of real-time PCR detection of phytoplasmas in infected tomatoes, potatoes, and beet leafhopper vectors. The procedure detected phytoplasmas in diverse phytoplasma groups (16SrI, VI, and IX) and thus may be suitable for "broad spectrum" phytoplasma detection. For example, this technique could be used for detection of AY phytoplasma in *Macrostelus* spp. vectors. Similarly, Christensen et al. (7) reported real-time PCR detection of diverse groups of phytoplasmas in infected periwinkles, including lucerne virescence, a member of group 16SrVI, using real-time PCR.

In tests conducted here, the concentration of phytoplasma varied greatly among purple top-symptomatic potato plants and beet leafhopper vectors. Similarly, by using competitive PCR, Berges et al. (3) reported that the concentration of aster yellows phytoplasma varied considerably among host plants. In real-time PCR experiments reported recently (20), the concentration of apple proliferation phytoplasma in different plants and the psyllid vector were found to vary by up to five orders of magnitude.

The ability to detect phytoplasmas in individual leafhopper vectors can aid in the identification of potential vector species (4,9,24). Additionally, the direct detection of phytoplasmas in vector insects may be a better predictor of possible disease problems than the insect population density alone (15). Detection of a pathogen within an insect, however, does not prove it is a vector, as was shown by Vega et al. (44). Previously, (9) PCR was used to demonstrate that of 15 leafhopper taxa tested, BLTVA was detected in only two, *C. tenel-*

Table 3. Real-time polymerase chain reaction (PCR) results with individual and groups of five beet leafhoppers (R^2 0.997441, slope -3.388 , threshold 1.0)^a

Sample	DNA concentration	Ct ^b	Quantity ^c	Nested PCR ^d	
BLTVA-infected tomato ^e	50 ng	26.02	50	+	
Healthy tomato ^f	50 ng	-- ^g	--	-	
Infected 5 beet leafhoppers ^h	50 ng	26.80	28.37	+	
	5 ng	30.23	2.76	+	
	500 pg	33.52	0.29	+	
	50 pg	37.38	0.02	-	
	5 pg	--	--	-	
Unknown 5 beet leafhoppers ⁱ	1	25 ng	36.58	0.04	-
	2	25 ng	29.45	4.68	+
	3	25 ng	30.05	3.12	+
	4	25 ng	--	--	-
	5	25 ng	--	--	-
	6	25 ng	--	--	-
	7	25 ng	30.06	3.10	+
	8	25 ng	30.64	2.09	+
	9	25 ng	31.14	1.48	+
	10	25 ng	30.56	2.20	+
	11	25 ng	28.94	6.61	+
	12	25 ng	28.85	7.03	+
	13	25 ng	--	--	-
	14	25 ng	--	--	-
	15	25 ng	--	--	-
	16	25 ng	30.52	2.26	+
	17	25 ng	35.62	0.07	+
	18	25 ng	--	--	-
	19	25 ng	24.41	144.60	+
	20	25 ng	28.24	10.71	+
	21	25 ng	--	--	-
	22	25 ng	29.16	5.71	+
	23	25 ng	--	--	-
Unknown single beet leafhopper samples	1	25 ng	28.62	8.24	+
	2	25 ng	39.15	0.01	-
	3	25 ng	30.91	1.74	+
	4	25 ng	30.97	1.67	+
	5	25 ng	--	--	-
	6	25 ng	--	--	-
	7	25 ng	--	--	-
	8	25 ng	--	--	-
	9	25 ng	--	--	-
	10	25 ng	--	--	-
	11	25 ng	--	--	-
	12	25 ng	35.12	0.10	+
	13	25 ng	28.01	12.49	+
	14	25 ng	--	--	+
	15	25 ng	--	--	-
	16	25 ng	--	--	-
	17	25 ng	--	--	-
	18	25 ng	--	--	-
	19	25 ng	--	--	-
	20	25 ng	--	--	-

^a Real-time PCR used primers 16Sp303F and 16Sp378R and probe 16TM329.

^b Threshold cycle.

^c Quantity is expressed as ng of phytoplasma-infected tomato DNA.

^d Nested PCR primers were P1/P7//FUS/BLTVA-int. (+) and (-) indicate that the expected band was present or absent, respectively, after agarose gel electrophoresis.

^e Beet leafhopper-transmitted virescence agent. Tested in triplicate. SD = 0.035.

^f Tested in triplicate. SD = N/A.

^g Not detected.

^h Tested in duplicate. SD = 0.000, 0.130, 0.077, 0.018, and N/A for 50 ng, 5 ng, 500 pg, 50 pg, and 5 pg, respectively.

ⁱ Single tests.

lus and less frequently in *Ceratagallia* spp. The nested PCR and real-time PCR tests of individual beet leafhoppers in this study indicated that 30% of the vectors contained detectable phytoplasma. This is approximately twice the percentage of phytoplasma-positive individual *C. tenellus* reported earlier (9). Goodwin et al. (15) reported the detection of aster yellows phytoplasma in up to 29% of individual aster leafhoppers collected at some locations. In contrast, Hill and Sinclair (17) detected phytoplasmas associated with ash yellows in only about 2% of potential leafhopper vectors.

Use of the real-time PCR methodology described herein, compared with the more commonly used nested PCR, will facilitate the testing of numerous insects collected from a commercial field setting and can rapidly determine whether or not they carry phytoplasma and therefore pose a threat to crop plants.

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