Improved Real-Time PCR Diagnosis of Citrus Stubborn Disease by Targeting Prophage Genes of *Spiroplasma citri*

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Abstract

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Spiroplasma citri is a phloem-limited bacterium causing citrus stubborn disease (CSD). Isolation and culturing of *S. citri* is technically demanding and time consuming. *S. citri* is typically low in titer and unevenly distributed in citrus, making reliable detection challenging. The current preferred detection method is polymerase chain reaction (PCR) assays with primers developed from sequences of *S. citri* housekeeping genes. Recent genome sequencing of *S. citri* revealed that the bacterium harbors multiple copies of prophage genes. Therefore, targeting multicopy prophage genes was hypothesized to improve sensitivity of PCR detection. Two primer sets, Php-orf1 and Php-orf3, were developed from conserved prophage sequences in the *S. citri* genome. These primer sets were used to evaluate detection sensitivity in SYBR Green-based quantitative PCR (qPCR) assays with 18 *S. citri* in cultures isolated from different hosts and locations. Prophage primer set

Php-orf1 increased detection sensitivity by 4.91 and 3.65 cycle threshold (Cq) units compared with housekeeping gene primers for spiralin and P58 putative adhesin gene, respectively. Detection was slightly less sensitive for the Php-orf3 primer set at 3.02 and 1.76 Cq units, respectively, over the same housekeeping gene primers. The prophage primer sets were validated for qPCR detection with field samples from three citrus orchards in California's San Joaquin Valley collected from 2007 to 2013. The data showed that *S. citri* prophage sequences improved sensitivity for qPCR detection of *S. citri*-infected trees at least 10-fold and reduced the number of false-negative results. No false-positive samples were detected with any of the primer sets. The enhanced sensitivity resulted from the higher copy number of prophage genes in the *S. citri* genome and, thus, improved CSD diagnosis from field samples.

Spiroplasma citri is a fastidious wall-less bacterium in the class Mollicutes. The bacterium infects citrus, causing citrus stubborn disease (CSD) in California, Arizona, North Africa, and the Mediterranean Basin (3). *S. citri* also has a wide host of nonrutaceous plant species (9,19). Recently, *S. citri* was reported to have been isolated from infected periwinkle in Malaysia (20). *S. citri* in citrus is spread by using infected budwood to propagate new trees or by transmission by several species of leafhoppers in a propagative manner (15,21). *S. citri* infection stunts citrus growth and decreases fruit production but does not kill trees. Typical symptoms include stunted growth of canopy, small leaves and bunched upright growth, chlorosis with zinc-like deficiency, premature fruit drop, low yield, and smaller or misshapen fruit (5). In California, CSD is a problem in the San Joaquin Valley and desert regions where hot, dry weather favors development of *S. citri* in various plant hosts and the beet leafhopper, *Circulifer tenellus*, principle vector of *S. citri* in this region (21).

Early detection of *S. citri* infection is important for CSD management. However, CSD is difficult to diagnose, especially in early

stages of disease development when symptoms are subtle or similar to other diseases or mineral deficiencies. Because *S. citri* isolation is technically demanding and time consuming, current detection is typically polymerase chain reaction (PCR)-based, using primers developed from sequences of housekeeping genes such as 16S ribosomal RNA (rRNA), spiralin, and the adhesin gene (12,29,30). Because of erratic distribution and low titer (9,29), *S. citri* DNA may not be reliably detectable by PCR using primers to sequences of low-copy genes in citrus.

To improve sensitivity of PCR detection, phage or prophage sequences of *S. citri* were used with good promise for detection in another study (12). The *S. citri* has the largest genome (1.6 to 1.9 Mbp) of all Mollicutes tested (24,27). This genome contains ubiquitous phage-related sequences such as plectrovirus SpV1, which was isolated and sequenced from *S. citri* R8A2 B (22). *S. citri* contained multiple dispersed copies of SpV1-related sequences which apparently play an important role in *S. citri* chromosomal rearrangement (16,27). More recently, a partial chromosome sequence of *S. citri* strain GII3-3X was reported and showed that phage-related and prophage sequences accounted for 20.5% of the sequenced genome (7).

Conserved multicopy DNAs provides higher template concentration for PCR detection. Therefore, it was hypothesized that PCR targeting phage or prophage genes may yield more sensitive detection than that from housekeeping genes. In this study, two primer sets, Php-orf1 and Php-orf3, were developed from prophage sequences in the *S. citri* genome. SYBR Green-based real time quantitative PCR (qPCR) was performed to evaluate detection sensitivity with *S. citri* cultures and validated with field samples from citrus orchards.

Materials and Methods

Bacterial strains. Eighteen *S. citri* strains were used in this study (Table 1). The source and isolation date were reported previously (17). Eleven of the strains were provided by Jacqueline

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Fletcher, Oklahoma State University, Stillwater, and are identified in Table 1 by a footnote. Cultivation was performed using standard procedures in LD8 medium (29) and strains were triply cloned and stored at -80°C (29). Primer specificity was evaluated using DNA extracted from the cultures as well as from infected plants harboring ‘*Candidatus Liberibacter asiaticus*’, ‘*Ca. L. solanacearum*’, *Xylella fastidiosa*, and *Xanthomonas citri* subsp. *citri* (Table 1).

Library preparation, Illumina sequencing, and assembly. Genomic DNA (50 ng) was fragmented with a modified transposition reaction called “tagmentation” with the Nextera DNA Sample Prep Kit (Epicentre Biotechnologies, Madison, WI). Eight different 6-bp barcoded adapters provided in the kit were used to prepare libraries compatible to Illumina sequencing. The resultant libraries, one for each strain of *S. citri*, were pooled together and sequenced on a single lane of the Illumina Genome Analyzer Iix at The University of Iowa. Two rounds of sequencing (72-bp single read and 2×72 -bp paired-end reads) were carried out followed by demultiplexing of data for each library into separate fastq files. A range of 633 Mb and 1.019 Gb of sequence data was generated for each strain. Assuming that the genome size of *S. citri* is 1.8 Mb (28), this accounts for $351\times$ to $566\times$ genome coverage. Each strain was assembled by combining the single and paired-end data using SOAPdenovo-V1.05 software (13). A range of K-mers was tried to generate optimal assemblies for each strain (Table 2).

Copy number analysis of the five genes. DNA libraries from the eight *S. citri* strains subjected to Illumina sequencing resulted in greater than 90% genome sequence for each of the eight strains (Table 2). These were compared with the complete genome of SpV1-R8A2 B (accession number X51344) and partial chromosome sequences of *S. citri* strain GII3-3X (accession number AM285301-AM285339) obtained from the GenBank database in the National Center for Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov/>). Due to the high abundance and multicopy sequences of SpV1-ORF1 and SpV1-ORF3, two phage-related genes in strain GII3-3X, these two genes were selected for further analysis (Table 3). The blast+ software package for standalone BLAST was downloaded from NCBI (6). 16S rRNA, spiralin gene, P58 gene, and two prophage genes were used as queries for local BLASTn search ($E < e^{-5}$) against contigs of eight *S. citri* strains and strain GII3-3X, respectively (Table 2).

Primer design. Two primer sets for qPCR were designed with Primer 3 software (23). Primers from spiralin and the putative adhesin gene (P58) gene were designed based on previous reports (29,30). All primer sets used in the study are listed in Table 3.

qPCR. qPCR assays based on SYBR Green included the newly developed Php-orf1 and Php-orf3 primer sets for sensitive detection and spiralin and P58 primer sets for comparison purposes. The reaction cocktail consisted of 25 μl of reaction mixture containing 12.5 μl of iQ SYBR Green Supermix (Bio-Rad), 0.2 μM each pri-

Table 1. Diagnostic sensitivity and specificity of real-time polymerase chain reaction base on four primers for the detection of *Spiroplasma citri* strains

Strain	Host	Origin	Cycle threshold values ^a			
			Spiralin	P58	Php-orf1	Php-orf3
<i>Spiroplasma citri</i> Ca1	Navel	Tulare County, CA	9.77	8.84	4.85	6.86
<i>S. citri</i> Ca16	Navel	Tulare County, CA	12.85	11.34	8.32	9.47
<i>S. citri</i> Ca263	Lambs quarter	Fresno County, CA	9.45	9.08	4.47	7.63
<i>S. citri</i> Ca264	Daikon	Fresno County, CA	8.16	6.87	3.46	5.28
<i>S. citri</i> CB1	BLH	Fresno County, CA	9.52	9.45	4.63	6.80
<i>S. citri</i> C5	Carrot	Kern County, CA	12.23	10.95	7.62	9.33
<i>S. citri</i> C17	Carrot	Kern County, CA	10.29	9.43	6.03	7.14
<i>S. citri</i> O202 ^b	Peach	California	10.02	8.41	4.96	6.41
<i>S. citri</i> O15 ^b	Broccoli	California	12.15	10.41	7.28	9.26
<i>S. citri</i> BR3 ^b	Horseradish	Illinois	9.31	8.64	4.66	6.67
<i>S. citri</i> BR6 ^b	Horseradish	Illinois	8.68	7.38	3.22	5.53
<i>S. citri</i> BR11 ^b	Horseradish	Illinois	10.59	8.63	5.38	7.98
<i>S. citri</i> MDHR2 ^b	Horseradish	Maryland	11.41	8.79	5.41	7.31
<i>S. citri</i> MDHR4 ^b	Horseradish	Maryland	8.99	6.66	3.70	5.07
<i>S. citri</i> R8A2 ^b	Sweet orange	Morocco	10.28	9.40	4.97	6.60
<i>S. citri</i> Beni ^b	Sweet orange	Morocco	12.02	11.93	8.37	10.05
<i>S. citri</i> Iran ^b	Sweet orange	Iran	11.71	9.28	6.74	7.98
<i>S. citri</i> ASP1 ^b	Sweet orange	Israel	8.00	7.23	3.01	5.71
‘ <i>Candidatus Liberibacter asiaticus</i> ’ GD-A4	Sweet orange	Guangdong, China	NA	NA	NA	NA
‘ <i>Ca. L. asiaticus</i> ’ CAHLB2012Las1	Pummelo/Lemon	Los Angeles County, CA	NA	NA	NA	NA
<i>Xanthomonas citri</i> subsp. <i>citri</i> XC62	Citrus sp.	Japan	NA	NA	NA	NA
<i>Xylella fastidiosa</i> M12	Almond	California	NA	NA	NA	NA
<i>X. fastidiosa</i> Dixon	Almond	California	NA	NA	NA	NA
‘ <i>Ca. L. solanacearum</i> ’	Potato	Riverside, CA	NA	NA	NA	NA

^a NA = not applicable.

^b Culture provided by Jaqueline Fletcher, Oklahoma State University, Stillwater. Original source material described by Mello et al. (17).

Table 2. Copy number of five genes in Illumina data from eight strains and submission sequences of GII3-3X^a

Strain	Total length (bp)	Contigs (>100 bp)	Contigs (N50)	Copy number				
				16S rRNA	Spiralin	P58	SpV1-ORF1	SpV1-ORF3
Ca1	1,860,679	3,375	59	1	1	5	54	18
Ca263	2,117,582	4,358	77	1	1	1	154	26
Ca264	2,381,867	6,324	223	1	1	3	115	8
CB1	1,692,836	2,686	60	1	1	3	56	19
C5	1,706,281	2,702	55	1	1	5	55	29
BR11	1,742,002	2,904	53	1	1	5	46	22
R8A2	1,994,329	4,097	134	1	1	5	93	35
Iran	2,450,939	5,836	270	1	3	1	140	56
GII3-3X	1,525,756 ^a	39	...	1	1	1	13	11

^a This is the nucleotide acid number deposited in the National Center for Biotechnology Information database. The estimated chromosome length of GII3-3X is 1,820 kbp (7).

mer, and 1 µl of DNA extract from field plants or *S. citri* cell culture DNA. Amplification, detection, and data analysis were performed with an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). The thermal profile consisted of one step at 95°C for 5 min, followed by 35 cycles of 95°C for 20 s, 58°C for 30 s, and 72°C for 30 s. Alternatively, a CFX96 Real-Time System with a C1000 thermal cycler (Bio-Rad Laboratories, Hercules, CA) was used where the thermal profile consisted of one step at 95°C for 3 min followed by 35 cycles of 95°C for 10 s and 59°C for 30 s.

qPCR sensitivity. To determine the analytic sensitivity and amplification efficiency of the qPCR assays, standard curves relating the amount of fluorescence to genomic DNA concentrations of *S. citri* per reaction were constructed using a series of 10-fold dilutions of LB10 cell culture DNA. *S. citri* was cultured in 25 ml of LD8 broth to a log stage and adjusted 20,000 cells/µl with a hemacytometer (C-Chip; Incyto, Covington, GA) with a dark-field compound microscope. DNA was extracted from an aliquot of 20,000 *S. citri* cells/µl by cetyltrimethylammonium bromide (CTAB) (8) and used at 1 µl per reaction for each 10-fold dilution up to 10⁻⁴. The DNA concentration was estimated to range from 64 pg to 0.064 fg per reaction. Three replicates of each dilution were tested simultaneously in the same run and four independent assays were performed. A linear relationship was produced by plotting the log DNA concentration against cycle threshold (Cq) value. qPCR amplification efficiency was estimated from the slopes of the standard curves using the equation $E = 10^{-1/\text{slope}} - 1$.

Field sample collection. To test and validate efficacy and sensitivity of the new primers, citrus fruit or leaves from three different orchards in the San Joaquin Valley were collected from the same plot trees from 2007 to 2013: (i) 22 *S. citri*-positive trees of 'Powell Navel' on 'Carrizo' citrange rootstock near Huron, Fresno County, CA; (ii) 51 *S. citri*-positive trees of 'Spring Navel' on Carrizo near Ducor, Tulare County, CA (Table 4); and (iii) 568 *S. citri*-positive and -negative trees of 'Cara Cara' on Carrizo near Mettler, Kern County, CA (Table 5). Leaves or fruit were harvested from all quadrant of each tree, labeled, and stored in ziplock bags at 4°C until processed. Purified DNA extracts were stored at -80°C until needed for the PCR testing. Primer set validation using Huron and Ducor trees included samples which previously were determined to be *S. citri* positive (30). The Mettler plot represented a second validation test in which the status of *S. citri* infection was unknown; thus, each tree in the plot was sampled and tested twice over a 2-year period.

DNA extraction. Fresh fruit columella tissue (200 mg) from three fruits or 10 leaf midribs per tree were excised and homogenized in a Homex 6 homogenizer (BioReba AG, Reinach, Switzerland). DNA was extracted by a CTAB method (8). A high-throughput extraction protocol was used for the 2013 plots from Mettler. A nucleic acid isolation kit (NucleoMag 96; Macherey-Nagel Inc., Bethlehem, PA) using magnetic beads was used in a BioSprint 96 (Qiagen Inc., Valencia, CA) instrument (25). DNA concentrations and purity were estimated by a NanoDrop ND-100 spectrophotometer (Wilmington, DE). Nucleic acid quality was determined using a plant cytochrome-oxidase-based primer-probe set as positive internal control to assess the quality of DNA extracts (14). Resultant Cq values were always between 17 and 19 for both CTAB and high-throughput extractions.

Statistical analysis. Analysis of variance (ANOVA) was performed on the means of Cq values using a single-factor ANOVA with a confidence level of $P = 0.05$.

Results

Illumina sequencing. Assembly of data resulted in 2,686 to 6,324 contigs of 100 bp or greater in length that were considered for further analyses. This accounts four 1.45 Mb to 1.85 Mb of assembled sequence in these strains (Table 2). The N50 contig size in these strains varied between 3.4 and 13.6 kb and the number of contigs in this N50 assembly varied between 53 and 270 contigs.

Estimation of gene copy numbers. Copy numbers of the five target genes in the eight *S. citri* genomes are shown in Table 2. Only one copy of 16S rRNA gene was identified in each of the eight genomes. Moreover, the spiralin gene was found as a single copy in seven of the genomes but was found with three copies in the Iranian strain, whereas the copy numbers of P58 gene ranged from one to five. In contrast, copy numbers of the two prophage genes were significantly higher, ranging from 54 to 154 for SpV1-ORF1 and 8 to 56 for SpV1-ORF3. The strain GII3-3X genome data was assembled into 39 contigs, whereas it was 53 to 270 for the other eight strains (Table 2).

Specificity and sensitivity analyses of four primer sets. In silico analysis indicated that the Php-orf3 primer set was specific for *S. citri*, whereas Php-orf1 matched with *S. citri* and *S. kunkelii*. qPCR specificity tests with primer sets Php-orf1, Php-orf3, spiralin, and P58 were evaluated with DNA extracts from cultures of 18 strains of *S. citri* originally obtained from a variety of hosts, including citrus, carrot, peach, broccoli, horseradish, and the beet leafhopper. In addition, extracts were also included from citrus bacteria '*Ca. L. asiaticus*' and *X. citri* subsp. *citri* and two other plant-associated bacteria: *Xylella fastidiosa* isolated from almond and '*Ca. L. solanacearum*' from potato. Amplicons were produced from all *S. citri* sources, whereas no amplicons from non-*S. citri* samples were obtained (Table 1).

Compared with primer sets based on housekeeping genes, prophage sequence primer set Php-orf1 increased Cq sensitivity of *S. citri* detection by an average of 4.91 ± 0.49 standard deviation (SD) and 3.65 ± 0.62 (SD) over spiralin and P58 gene primers, respectively. *S. citri* detection by Php-orf3 was slightly less sensi-

Table 4. Mean cycle threshold (Cq) values comparison of real-time polymerase chain reaction based on four primer sets from stored DNA from 73 *Spiroplasma citri*-positive citrus trees located in two commercial orchards in Huron (southwestern Fresno County) and Ducor (southeastern Tulare County), CA

Collection date	Average Cq values per primer set per year for <i>S. citri</i> detection ^a			
	Spiralin	P58	Php-orf1	Php-orf3
2007	26.08	24.75	21.64	22.98
2008	28.23	26.59	23.17	24.38
2009	28.61	27.39	24.97	26.68
2011	27.85	26.03	22.99	24.58
Average	27.69	26.19	23.19	24.66

^a There were 252 DNA samples extracted using cetyltrimethylammonium bromide method (8) and stored from 73 *S. citri*-positive trees collected annually over the 4-year sampling period.

Table 3. Primers used in real-time polymerase chain reaction for the detection of *Spiroplasma citri*

Primer name	Sequence (5'-3')	Target gene	Size	Copy number	Reference
Spiralin-F/R	AAGCAGTGCAAGGAGTTGTAAAAA/ TGATGTACCTTTGTGTCTTGATAAACA	Spiralin	79	1	30
P58-F/R	GTCCCTAATGCACCGTGAAAAA/ GCACAGCATTGCGCAACTACA	Putative adhesin gene	119	1	29
Php-orf1-F/R	TGGCAGTTTTGTGTTAGTCATCC/ GGGTCTAAACGCCGTTAAAGT	SpV1-ORF1	190	13	This study
Php-orf3-F/R	CTGGTTCAACCACAACAAAA/ AAACACGGTCGGAGTTTATCA	SpV1-ORF3	149	11	This study

tive than Php-orf1, with an average Cq of 3.02 ± 0.62 (SD) and 1.76 ± 0.51 (SD) over spiralin and P58, respectively. Significant differences were obtained using single-factor ANOVA of Cq values comparing spiralin and Php-orf3 ($P = 6.35 \times 10^{-7}$); and spiralin and Php-orf1 ($P = 6.59 \times 10^{-11}$). The Cq value differences between P58 and Php-orf3 or Php-orf1 were also significant ($P = 0.001$ and $P = 5.47 \times 10^{-8}$, respectively).

Detection limits and PCR amplification efficiency. Ten-fold serial dilutions of genomic DNA of *S. citri* strain LB10 showed high efficiencies of the prophage primer sets (Fig. 1). Standard curve equations of spiralin, P58, Php-orf1, and Php-orf3 primer sets were $Y_{\text{spiralin}} = -3.587X + 34.617$, $Y_{\text{P58}} = -3.544X + 29.816$, $Y_{\text{Php-orf1}} = -3.422X + 27.900$, and $Y_{\text{Php-orf3}} = -3.543X + 28.075$, respectively. The correlation coefficient of the four standard curves ranged from 0.997 to 1.00, thus indicating excellent association between template amount and Cq value. The PCR amplification efficiencies were 90.0, 91.5, 96.0, and 91.5%, respectively, for each of the primer sets. *S. citri* detection of DNA from bacterial cells was linear throughout the range of cells tested for all primer sets, indicating that conservative Cq cutoff values for diagnosis of a *S. citri*-positive sample were 35 and 31 for spiralin and prophage primer sets, respectively. The bacterial detection limits for spiralin and P58 primers were 6.4 fg, whereas that for the two prophage primers were 0.64 fg. No amplification was obtained when DNA concentration of the LB10 strain was below 0.64 fg per reaction or in the water only control.

Evaluation with CSD-affected field samples. In all, 252 *S. citri*-positive sample extracts had been stored at -80°C from 73 *S. citri*-infected trees from the Huron and Ducor plots (Table 4). These samples had been collected annually over a 4-year period (2007 to 2011) and used in this study. *S. citri* was detected in 240 (95.24%), 243 (96.43%), 250 (99.21%), and 249 (98.81%) extracts by spiralin, P58, Php-orf1, and Php-orf3 primer sets, respectively. The mean Cq values of field samples also indicated that the prophage primers had greater detection sensitivity than spiralin and P58 (Table 4). Because this analysis did not include samples from *S. citri*-negative trees, confirmation of false-negative samples from housekeeping gene primer sets was confirmed by positive tests with the two prophage primer sets. *S. citri* detection from field samples was also evaluated in another set of multiple-year samples from 568 field trees (Table 5). This analysis differed from the Huron or Ducor set because all trees sampled including those not infected were tested by primer sets spiralin and Php-orf1. In 2010, the DNA was extracted by CTAB and the spiralin primer set detected 102 infected trees; whereas Php-orf1 detected 108 trees. In 2013, the same trees were sampled but DNA was obtained by high-throughput extraction. The spiralin primers detected 106 infected trees while Php-orf1 detected 112. Thus, in 2010 and 2013, the prophage primer set found six additional infected trees (confirmed by a separate test with Php-orf3) compared with the spiralin primer

set. Based on redundant assays with primer sets from different gene regions, no false-positive results were detected with any of the primer sets. Therefore, the false readings using housekeeping genes were approximately 1% (6/568) and approximately 5% (6/112) if only positive samples were considered versus the prophage primer sets. The average Cq value for infected trees sampled in 2010 and DNA extracted by CTAB was 31.3 and 27.8 for spiralin and Php-orf1, respectively; and, in 2013 samples processed by high-throughput extraction, was 32.8 versus 26.8 for spiralin and Php-orf1, respectively.

Discussion

Current qPCR assays for *S. citri* target low-copy housekeeping genes of *S. citri*, which have the characteristics of high fidelity and specificity. However, due to the low ratio of *S. citri* DNA relative to background DNA within samples, detection of low titer levels of the pathogen can be unreliable. Alternatives such as nested PCR (10) or Bio-PCR (26) are not convenient for large-scale surveys due to increased handling and potential for contamination. Deep sequencing of *S. citri* strains extended the genomic information available for improved PCR detection of the pathogen. Phage-related sequences from *S. citri* strain GII3-3X were obtained from Illumina sequencing of eight *S. citri* strains (R. K. Yokomi, unpublished data). Because the single-copy nature of 16S rRNA gene in the nine strains examined was expected (7,28), sequence duplication in these genomes was minimal and predicted gene copy numbers were acceptable. The high copy numbers of the two prophage genes in the *S. citri* genomes sequenced showed that prophage genes in *S. citri* were ubiquitous and abundant. There are other reports of multiple copies of SpV1-type phages or at least contain SpV1-related sequences in chromosomal DNA of *S. citri* (2,7,27). Based on prophage genes with tandem repeats, Morgan et al. (18) developed a qPCR method which could improve detection sensitivity of '*Ca. L. asiaticus*'. In the present study, two newly developed primer sets, Php-orf1 and Php-orf3, were selected from two *S. citri* phage or prophage genes, SpV1-ORF1 and SpV1-ORF3. These two genes are annotated as a hypothetical protein and a putative transposase, respectively.

The prophage primers readily detected all *S. citri* strains tested and sensitivity of qPCR assays was improved more than 10-fold from that of spiralin or the putative P58 adhesin gene. The in silico match of Php-orf1 sequences with *S. citri* and *S. kunkelii*, causal agent of corn stunt disease (4), exemplifies the risk of false positives with the exclusive use of nonhousekeeping genes for PCR detection of the pathogen. As was done in this study, primer sets to several conserved housekeeping genes should be included to confirm PCR detection.

Primer sets Php-orf1 and Php-orf3 resulted in significantly lower mean Cq values regardless of how the target DNA was acquired. Use of the prophage primers in qPCR assays with field samples

Table 5. Cycle threshold (Cq) values to detect *Spiroplasma citri*-infected citrus trees by real-time polymerase chain reaction comparing primer sets of spiralin versus Php-orf1 with SYBR Green

Plot ^d	Number of trees	Number of <i>S. citri</i> -positive Cq values ^a			
		2010 CTAB ^b		2013 HTE ^c	
		Spiralin	Php-orf1	Spiralin	Php-orf1
1	256	37	37	40	39
2	64	12	15	16	16
3	64	19	20	19	19
4	57	5	5	6	10
5	63	7	9	8	9
6	64	23	22	17	19
Totals	568	102	108	106	112
Infection (%)	...	18.0	18.7	18.7	19.7
Average Cq	...	31.3 $\pm$ 2.0	27.8 $\pm$ 3.2	32.8 $\pm$ 2.0	26.8 $\pm$ 2.3

^a Conservative cut off Cq values for positive diagnosis of *S. citri*-infection in citrus tissue samples were 35 and 31 for spiralin and prophage primers, respectively.

^b Target DNA extraction (2010) by cetyltrimethylammonium bromide (CTAB) method (8).

^c Target DNA extraction (2013) by high-throughput extraction (HTE) (25).

^d Citrus cultivar in plot was Cara Cara on Carrizo rootstock planted in 2007 near Mettler, Kern County, CA.

showed its value by detecting *S. citri* infection from samples previously evaluated as negative by qPCR with housekeeping genes. Reducing the risk of false negatives is critically important if PCR diagnosis of *S. citri* infection is used in certification programs.

Due to the abundance of repeated sequences, many related to phages or prophages, closure of the *S. citri* chromosome was not

successful (7,24). Recently, the complete genomes of *S. chrysopicola* and *S. syriphidicola* were reported (11). Interestingly, these two species did not contain pleuroviral genes. With regard to the virus-free genomes, further analyses suggested that phage invasions were critical for horizontal gene transfers that contributed to the bacterial environmental adaptation (11). Nevertheless, data

Fig. 1. Standard curve of cycle threshold (Cq) values of 10-fold dilution DNA extracted from *Spiroplasma citri* (LB10) at 20,000 cells/ μ l in quantitative polymerase chain reaction assays with the different gene primer sets. Each reaction contained 1 μ l of cell suspension and the reporting dye was SYBR Green. **A**, Spiralin; **B**, P58; **C**, Php-orf-1; and **D**, Php-orf-3. RFU = relative fluorescence units.

mining from additional sequencing efforts will be helpful to screening additional genomic loci with potential to further improve detection of *S. citri*. It is interesting to note that the primer set Php-orf1 could target the gene in *S. kunkelii* via in silico analysis, suggesting that it also has the potential to improve detection of *S. kunkelii*, in which the plectroviral genes were found to be abundant (1,31).

In conclusion, qPCR targeting high-copy-number genes such as SpV1-ORF1 significantly improved *S. citri* detection in culture and field samples. These results were validated by side-by-side qPCR using housekeeping and prophage gene primers. The increased sensitivity has additional value for early detection of the disease agent. The addition of automated high-throughput DNA extraction increased laboratory processing efficiency, reduced pipetting errors, and improved standardization needed for large-scale field surveys for epidemiology studies.

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Literature Cited

- Bai, X., and Hogenhout, S. A. 2002. A genome sequence survey of the mollicute corn stunt spiroplasma *Spiroplasma kunkelii*. FEMS Microbiol. Lett. 210:7-17.
- Bébéar, C. M., Aulio, P., Bové, J. M., and Renaudin, J. 1996. *Spiroplasma citri* virus SpV1: Characterization of viral sequences present in the spiroplasma host chromosome. Curr. Microbiol. 32:134-140.
- Bové, J. M., and Garnier, M. 2000. Stubborn. Pages 48-50 in: Compendium of Citrus Diseases. L. W. Timmer, S. M. Garnsey, and J. H. Graham, eds. American Phytopathological Society, St. Paul, MN.
- Bradford, O. E., Tsai, J. H., and Gordon, D. T. 1981. Corn stunt spiroplasma and viruses associated with a maize disease epidemic in southern Florida. Plant Dis. 65:837-841.
- Calavan, E. C., and Oldfield, G. N. 1979. Symptomatology of spiroplasma plant disease. Pages 37-64 in: The Mycoplasmas. R. F. Whitcomb and J. G. Tully, eds. Academic Press, Inc., New York.
- Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., and Madden, T. L. 2009. BLAST+: Architecture and applications. BMC Bioinf. 10:421.
- Carle, P., Saillard, C., Carrère, N., Carrère, S., Duret, S., Eveillard, S., Gaurivaud, P., Gourgues, G., Gouzy, J., Salar, P., Verdin, E., Breton, M., Blanchard, A., Laigret, F., Bové, J. M., Renaudin, J., and Foissac, X., 2010. Partial chromosome sequence of *Spiroplasma citri* reveals extensive viral invasion and important gene decay. Appl. Environ. Microbiol. 76:3420-3426.
- Doyle, J. J. 1991. DNA protocols for plants. Pages 283-293 in: Molecular Techniques in Taxonomy. G. Hewitt, A. W. B. Johnson, and J. P. W. Young, eds. NATO ASI Ser. H, Cell Biol. 57. Springer-Verlag, Berlin.
- Gumpf, D. J., and Calavan, E. C. 1981. Stubborn disease of citrus. Pages 97-134 in: Mycoplasma Diseases of Trees and Shrubs. K. Maramorosch and S. P. Raychaudhuri, eds. Academic Press, New York.
- Haff, L. A. 1994. Improved quantitative PCR using nested primers. Genome Res. 3:332-337.
- Ku, C., Lo, W. S., Chen, L. L., and Kuo, C. H. 2013. Complete genomes of two dipteran-associated spiroplasmas provided insights into the origin, dynamics, and impacts of viral invasion in *Spiroplasma*. Genome Biol. Evol. 5:1151-1164.
- Lee, I. M., Bottner, K. D., Munyaneza, J. E., Davis, R. E., Crosslin, J. M., du Toit, L. J., and Crosby, T. 2006. Carrot purple leaf: A new spiroplasma disease associated with carrots in Washington State. Plant Dis. 90:989-993.
- Li, R., Li, Y., Kristiansen, K., and Wang, J. 2008. SOAP: Short Oligonucleotide Alignment Program. Bioinformatics 24:713-714.
- Li, W., Hartung, J. S., and Levy, L. 2006. Quantitative real-time PCR for detection and identification of 'Candidatus Liberibacter' species associated with citrus huanglongbing. J. Microbiol. Methods 66:104-115.
- Liu, H.-Y., Gumpf, D. J., Oldfield, G. N., and Calavan, E. C. 1983. The relationship of *Spiroplasma citri* and *Circulifer tenellus*. Phytopathology 73:585-590.
- Melcher, U., Sha, Y., Ye, F. C., and Fletcher, J. 1999. Mechanisms of *Spiroplasma* genome variation associated with SpV1-like viral DNA inferred from sequence comparisons. Microb. Comp. Genomics 4:29-46.
- Mello, A. F. S., Yokomi, R. K., Melcher, U., Chen, J. C., Wayadande, A. C., and Fletcher, J. 2008. Genetic diversity of *Spiroplasma citri* strains from different regions, hosts, and isolation dates. Phytopathology 98:960-968.
- Morgan, J. K., Zhou, L. J., Li, W., Shatters, R. G., Keremane, M., and Duan, Y. P. 2012. Improved real-time PCR detection of 'Candidatus Liberibacter asiaticus' from citrus and psyllid hosts by targeting the intragenic tandem-repeats of its prophage genes. Mol. Cell. Probes 26:90-98.
- Nejat, N., Vadamalai, G., and Dickinson, M. 2011. *Spiroplasma citri*: A wide host range phytopathogen. Plant Pathol. J. 10:46-56.
- Nejat, N., Vadamalai, G., Sijam, K., and Dickinson, M. 2011. First report of *Spiroplasma citri* associated with periwinkle lethal yellows in Southeast Asia. Plant Dis. 95:1312.
- Oldfield, G. N., Kaloostian, G. L., Pierce, H. D., Calavan, E. C., Granett, A. L., Blue, R. L., Rana, G. L., and Gumpf, D. J. 1977. Transmission of *Spiroplasma citri* from citrus to citrus by *Scaphytopius nitridus*. Phytopathology 67:763-765.
- Renaudin, J., Aulio, P., Vignault, J. C., and Bové, J. M. 1990. Complete nucleotide sequence of the genome of *Spiroplasma citri* virus SpV1-R8A2 B. Nucleic Acids Res. 18:5.
- Rozen, S., and Skaletsky, H. J. 2000. Primer 3 on the WWW for general users and for biological programmers. Methods Mol. Biol. 132:365-386.
- Saillard, C., Carle, P., Duret-Nurbel, S., Henri, R., Killiny, N., Carrère, S., Gouzy, J., Bové, J. M., Renaudin, J., and Foissac, X. 2008. The abundant extrachromosomal content of *Spiroplasma citri* strain GII3-3X. BMC Genomics 9:195-207.
- Saponari, M., Loconsole, G., Liao, H.-H., Jiang, B., Savino, V., and Yokomi, R. K. 2013. Validation of high-throughput real time polymerase chain reaction assays for simultaneous detection of invasive citrus pathogens. J. Virol. Methods 193:478-486.
- Schaad, N. W., Cheong, S. S., Tamaki, S., Hatziloukas, E., and Panopoulos, N. J. 1995. A combined biological and enzymatic amplification (BIO-PCR) technique to detect *Pseudomonas syringae* pv. *phaseolicola* in bean seed extracts. Phytopathology 85:243-248.
- Ye, F. C., Laigret, F., Carle, P., and Bové, J. M. 1995. Chromosomal heterogeneity among various strains of *Spiroplasma citri*. Int. J. Syst. Bacteriol. 45:729-734.
- Ye, F. C., Laigret, F., Whitley, J. C., Citti, C., Finch, L. R., Carle, P., Renaudin, J., and Bové, J. M. 1992. A physical and genetic map of the *Spiroplasma citri* genome. Nucleic Acids Res. 20:1559-1565.
- Yokomi, R. K., Mello, A. F. S., Saponari, M., and Fletcher, J. 2008. Polymerase chain reaction-based detection of *Spiroplasma citri* associated with citrus stubborn disease. Plant Dis. 92:253-260.
- Yokomi, R. K., and Sisterson, M. 2011. Validation and comparison of a hierarchical sampling plan for estimating incidence of citrus stubborn disease. In: Proc. 18th Conf. Int. Organ. Citrus Virol. IOCV, Riverside, CA. http://iocv.org/proceedings/eighteen/Yokomi_and_Sisterson.pdf
- Zhao, Y., Hammond, R. W., Jomantiene, R., Dally, E. L., Lee, I. M., Jia, H., Wu, H., Lin, S., Zhang, P., Kenton, S., Najjar, F. Z., Hua, A., Roe, B. A., Fletcher, J., and Davis, R. E. 2003. Gene content and organization of an 85-kb DNA segment from the genome of the phytopathogenic mollicute *Spiroplasma kunkelii*. Mol. Genet. Genomics 269:592-602.