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Specific PCR Detection and Identification of *Xylella fastidiosa* Strains Causing Citrus Variegated Chlorosis

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Abstract. By cloning and sequencing specific randomly amplified polymorphic DNA (RAPD) products, we have developed pairs of PCR primers that can be used to detect *Xylella fastidiosa* in general, and *X. fastidiosa* that cause citrus variegated chlorosis (CVC) specifically. We also identified a CVC-specific region of the *X. fastidiosa* genome that contains a 28-nucleotide insertion, and single base changes that distinguish CVC and grape *X. fastidiosa* strains. When using RAPD products to develop specific PCR primers, we found it most efficient to screen for size differences among RAPD products rather than presence/absence of a specific RAPD band.

Citrus variegated chlorosis (CVC), first observed in Brazil in 1987 [19], is a disease of sweet orange (*Citrus sinensis* L.) caused by *Xylella fastidiosa* [6, 10] that severely limits usable fruit yield. Disease symptoms include mottled chlorosis, reduced leaf size, and fruit that are small, early maturing, and have a very hard rind which can damage juicing machines, resulting in rejection of entire lots of fruit at juicing plants [13]. Transmission can occur through grafting in commercial nurseries, or through sharpshooter leafhopper vectors (*Homalodiscus* and *Oncometopia* spp.) [5]. Although CVC is presently localized to the states of Minas Gerais and Sao Paulo in southeastern Brazil, it is a potential threat to all citrus growing regions of the world. Epidemiological studies estimate that CVC from a single infected tree can spread to 90% of the trees in a 20 hectare grove in 12 years [8]. Limiting the spread of CVC requires sensitive detection techniques to prevent the introduction of the CVC-causing *X. fastidiosa* bacterium into noninfected areas or countries through infected budwood. Traditional methods for detecting phytopathogenic bacteria rely on the isolation and culture of the bacteria followed by serological testing. Such methods are especially difficult with *X. fastidiosa* owing to its fastidious nutritional requirements and extremely slow growth in culture. Recently a protocol was developed that allows detection of *X. fastidiosa* from plant tissue on the basis of the polymerase chain reaction (PCR) by use of *X. fastidiosa*-specific primers [14]. In theory, such a method allows detection of as few as 10–100 bacteria per amplification reaction. We have cloned and sequenced specific RAPD-PCR products and have developed a set of PCR primers that are specific only to *X. fastidiosa* strains causing CVC.

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Materials and Methods

Bacterial strains and DNA extraction. Five strains of *Xylella fastidiosa* were isolated in Beltsville, Maryland from twigs collected by J. Beretta (Instituto Biologico, Sao Paulo) from CVC-infected sweet orange 'pera' grown in Bebedouro, Sao Paulo, Brazil. Two strains were isolated in Brazil by J. Beretta, three strains were isolated in Florida by D. Hopkins (University of Florida, Leesburg), and 11 strains isolated from diverse woody plants in North America were obtained from the American Type Culture Collection, Rockville, Maryland (Table 1). This collection was screened to identify RAPD-PCR products specific to the citrus strains of *X. fastidiosa*. For comparison, several bacterial strains from other plant-associated genera were also screened by RAPD-PCR, including *Xanthomonas campestris* pv. *citri* strain Xc102 (causal agent of citrus bacterial canker) [9], *Erwinia amylovora* strain Ea110R from apple (causal agent of fire blight) [17], *Enterobacter cloacae* strain 501R3 from cotton (plant rhizosphere bacterium) [18], and *Escherichia coli* strain HB101 [4]. *Xylella fastidiosa* strains were cultured in PW broth [7] supplemented with 5 g malt extract, 10 g sucrose, 100 mg myoinositol, 10 mg thiamine HCl, 10 mg pyridoxine, 5 mg nicotinic acid, and 2 mg glycine per liter [10]. Cultures were maintained in the dark at 28°C for 9–14 days. *Xanthomonas campestris*, *E. cloacae*, *E. amylovora*, and *E. coli* were grown

Table 1. Strain, host, and origin of the *Xylella fastidiosa* analyzed in this study

Strain	Host	Origin[a]
CVC93-1-r	citrus	Brazil (B)
CVC94-2	citrus	Brazil (B)
CVC-PW	citrus	Brazil (B)
CVC102	citrus	Brazil (B)
CVC#5	citrus	Brazil (B)
Xf-1	citrus	Brazil (JB)
Xf-2	citrus	Brazil (JB)
CB9	citrus	Florida (DH)
PD88-5A	grape	Florida (DH)
PD92-8	grape	Florida (DH)
ATCC 35868	mulberry	Massachusetts (A)
ATCC 35869	mulberry	Massachusetts (A)
ATCC 35870	almond	California (A)
ATCC 35871	plum	Georgia (A)
ATCC 35873	elm	Washington, DC (A)
ATCC 35874	oak	Washington, DC (A)
ATCC 35876	ragweed	Florida (A)
ATCC 35877	grapevine	California (A)
ATCC 35878	periwinkle	Florida (A)
ATCC 35879	grapevine	Florida (A)
ATCC 35881	grapevine	Florida (A)

[a] *X. fastidiosa* strains isolated in Beltsville, Maryland (B); isolated in Brazil by Julia Beretta (JB); isolated in Florida by Don Hopkins (DH); or obtained from the American Type Culture Collection, Rockville, Maryland (A).

overnight in LB medium [2]. Bacterial genomic DNA extractions were performed as described by Ausubel et al. [2]. DNA concentration and purity were assessed by comparison with known standards on an ethidium bromide-stained 1% agarose gel.

RAPD reaction conditions. Randomly amplified polymorphic DNA technology (RAPD [20]) was used to identify PCR products unique to the CVC-causing strains of *X. fastidiosa*. RAPD-PCR was performed in a 25-μl volume containing PCR buffer (20 mM NaCl, 50 mM Tris pH 9.0, 1% Triton X-100, 0.1% gelatin, 3 mM $MgCl_2$ [3]), 200 μM dNTP, 0.2 μM 10-base primer (UBC set #3 from University of British Columbia Nucleic Acid-Protein Service Unit, Vancouver, BC, Canada), 50 ng template DNA, and 0.5 unit Taq DNA Polymerase (Life Technologies, Grand Island, NY). PCR reactions were performed in a Thermal Cycler (Perkin Elmer, Norwalk, Connecticut) using the following profile: 45 cycles of 1 min at 94°C, 1 min at 48°C, and 2 min at 72°C. Completed reactions were maintained at 4°C until analysis on 1.5% agarose TAE gels visualized with ethidium bromide.

DNA purification, cloning, and sequencing. RAPD bands of interest were purified from agarose gels with Qiaex resin (Qiagen, Chatsworth, California). Purified DNA was cloned into the TA cloning vector (Invitrogen, San Diego, California) according to the manufacturer's instructions, and the plasmid was introduced into competent *E. coli* strain DH5α (Life Technologies). Plasmid DNA was isolated from *E. coli* by use of Qiagen columns according to the manufacturer's recommendations, and the inserts were partially sequenced with ^{35}S-dATP and the Sequenase version 2.0 protocol for plasmid DNA templates (US Biochemical, Cleveland, Ohio).

Primers and PCR reaction conditions. Oligonucleotide primers were manufactured by Cruachem (Dulles, Virginia). All primers except 272-1-int, 272-2-int, and CVC-1 consisted of the original 10-base RAPD primer plus the next ten or 11 nucleotides as determined by sequence analysis (Table 2).

PCR was performed in a 25-μl volume containing the buffer described previously, 200 μM dNTP, 0.4 μM primer, and 1.0 unit Taq DNA polymerase. Five thousand to 10,000 bacteria as estimated by hemacytometer counts, or 1–5 μl of vacuum-extracted xylem sap from a CVC-infected sweet orange tree was added to the reaction. DNA was amplified by lysing bacteria at 94°C for 4 min, followed by an amplification profile of 94°C for 1 min, 60–72°C for 1 min (see Table 2 for annealing temperatures), 72°C for 1 min for 30 cycles, followed by a final extension at 72°C for 10 min. PCR products were analyzed by agarose gel electrophoresis and visualized with ethidium bromide.

Results and Discussion

Our laboratory previously demonstrated that RAPD-PCR detected differences among *X. fastidiosa* strains and indicated that strains of *X. fastidiosa* causing CVC were only distantly related to the other *X. fastidiosa* strains as evidenced by many non-shared RAPD products [16]. On the basis of these results, we concluded that RAPD-PCR products unique to strains of *X. fastidiosa* causing CVC could be used as the basis for developing CVC-specific PCR primers. A similar approach was successfully used in lettuce to develop PCR-based markers linked to downy mildew resistance genes, and these markers were described as sequence-characterized, amplified regions or SCARs [15]. Four unique RAPD products, each specific to CVC strains of *X. fastidiosa*, were amplified with UBC primers 203, 204, 208, and 230, and were subsequently cloned and partially sequenced. RAPD sequence information provided the basis for the selection of several pairs of 20-base primers (Table 2). However, when standard PCR assays were performed with these new primers at an annealing temperature corresponding to the lowest melting temperature of each pair, we found that the primers amplified an identical band in all strains of *X. fastidiosa*, including non-CVC strains, even when no such band was amplified by RAPD-PCR (Fig. 1). Similar results were also observed in lettuce [15], where six out of nine RAPD-derived 24-base PCR primers amplified DNA in individuals that did not have the corresponding RAPD product. This result can be explained if a primer-template mismatch near the 5′ end of the 10-nucleotide primer determined the original RAPD polymorphism. Such a phenomenon could result in a primer-template mismatch near the 5′ end that was tolerated by the 20-base primer and therefore allowed priming to continue from the 3′ end. Thus, although the strains may have differed in the original 10-base priming sites, these differences were not detected with specific 20-base

Table 2. List of primers, annealing temperatures, size of PCR products, and strain specificity

Primer pair[a]	Sequence[b] 5′–3′	Annealing Temp (°C)	Approximate size of PCR product (bp)	Strain specificity[c]
208-1	ACGGCCGACCATTACTGCTG	70	320	All *X. fastidiosa*
208-2	ACGGCCGACCCGGAGTATCA			
203-1	CACGGCGAGTATCGGCTTTC	67	2000	All *X. fastidiosa*
203-2	CACGGCGAGTCGACGTAAAT			
204-1	TTCGGGCCGTCAATGTGTTG	66	800	All *X. fastidiosa*
204-2	TTCGGGCCGTTTACTCAAAG			
230-1	CGTCGCCCATCAACGCCAAA	72	700	All *X. fastidiosa*
230-2	CGTCGCCCATTGTGCTGCAG			
272-1	AGCGGGCCAATATTCAATTGC	68	700	All *X. fastidiosa* + X.c. + E.a.
272-2	AGCGGGCCAAAACGATGCGTG			
272-1-int	CTGCACTTACCCAATGCATCG	67	500	All *X. fastidiosa*
272-2-int	GCCGCTTCGGAGAGCATTCCT			
CVC-1	AGATGAAAACAATCATGCAAA	62	600	CVC *X. fastidiosa* + X.c. + E.a.
272-2	AGCGGGCCAAAACGATGCGTG			
CVC-1	AGATGAAAACAATCATGCAAA	62	500	CVC *X. fastidiosa*
272-2-int	GCCGCTTCGGAGAGCATTCCT			

[a] Except for primer CVC-1, all primers are named for the UBC 10-base primer that produced the differential RAPD products.
[b] Except for primer CVC-1, 272-1-int, and 272-2-int, the first 10 nucleotides in the sequence (underlined) correspond to the sequence of the original UBC 10-base RAPD primer.
[c] Primer-amplified product only from tested strains of *X. fastidiosa;* from *X. fastidiosa* and strains of *Xanthomonas campestris* (X.c.) and *Erwinia amylovora* (E.a.), or from CVC-specific strains of *X. fastidiosa.*

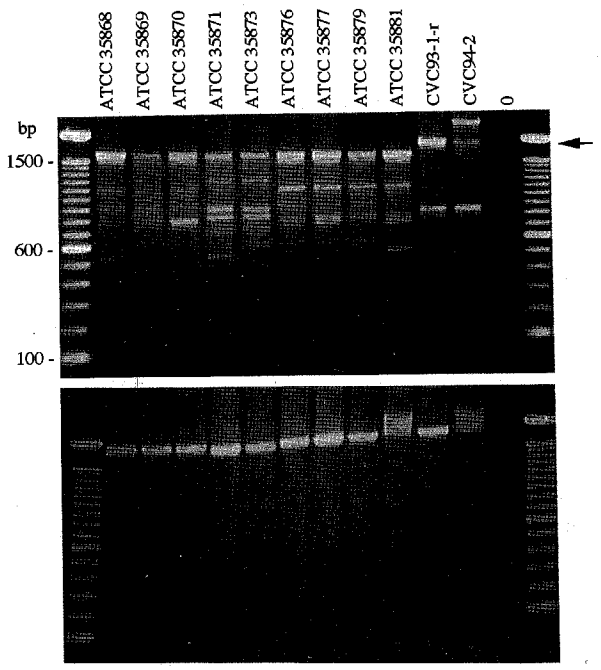

Fig. 1. Amplification of *X. fastidiosa* genomic DNA with 10-base RAPD primer UBC #203 (top gel) or with *X. fastidiosa*-specific 20-base PCR primers 203-1 and 203-2 (bottom gel). The outside lanes contain 100-bp marker DNA. The first nine lanes are ATCC strains, the next two lanes are CVC strains (see Table 1 for host), and the last lane contains the no-template control. Note the 2000-bp DNA product (arrow) is amplified only in CVC strains with the 10-nucleotide RAPD primer, but is amplified in all *X. fastidiosa* strains with the 20-base primers.

primers. This observation is supported by evidence from RAPD hybridization studies in *Helicobacter pylori* [1], in which most polymorphisms were caused by differences in priming sites rather than insertions or deletions.

Another RAPD-derived primer pair, 272-1 and 272-2, amplified an approximately 700 base pair product in all *X. fastidiosa* strains examined, as well as faint bands in *Xanthomonas campestris* and *Erwinia amylovora*. However, upon closer analysis, the amplified DNA from the CVC-specific strains appeared to be larger than the other *X. fastidiosa* strains, as indicated by slightly slower migration on a 1.5% agarose gel (not shown). Sequence comparison of the amplified products from CVC strain 94-2 and grape strain 35877 revealed that the CVC strain contained a 28-nucleotide insertion that occurred 30 bases downstream of the original 21-base 272-1 primer (Fig. 2). Because this insertion was not present in the non-CVC strains, it was successfully used as the basis to construct a CVC-specific primer. Thus, although insertions and deletions are harder to detect in bacterial genomes [1], we have concluded that screening bacterial strains for RAPD product size differences rather than presence/absence of a RAPD product is the most efficient method to develop specific RAPD-derived PCR primers in *X. fastidiosa*. From sequence information internal to the CVC-1 and 272-2 primers, we also constructed primers spe-

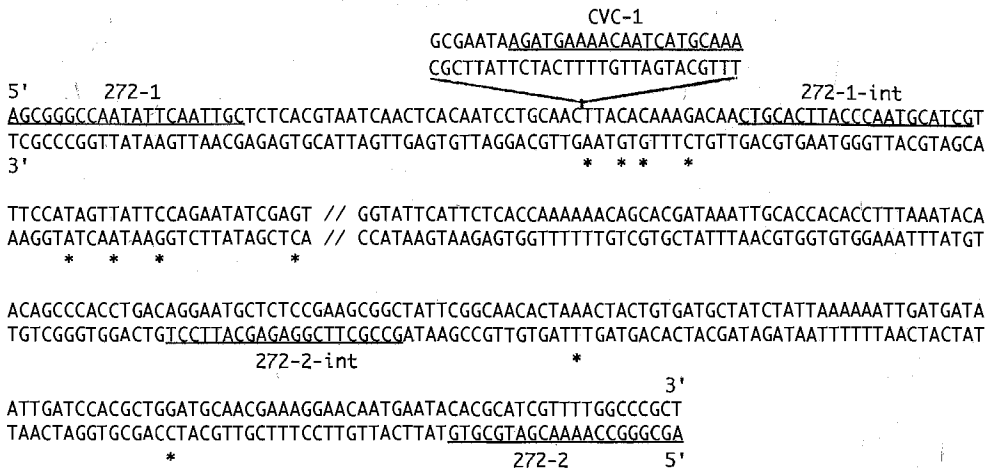

Fig. 2. Partial sequence of the cloned 700-bp product from *Xylella fastidiosa* strain CVC94-2 amplified by RAPD primer 272. Oligonucleotide primers (see Table 2) are underlined. The 28-nucleotide insertion, which is present only in CVC strains, was used to construct the CVC-specific primer, CVC-1. Asterisks denote nucleotides that differ in the grape 35877 strain.

cific to *X. fastidiosa* that did not amplify DNA from *X. campestris* or *E. amylovora* (Table 2).

Interestingly, the sequence following the CVC insertion revealed eight out of 63 nucleotide changes between the citrus CVC 94-2 and grape 35877 strains (50% transversions). Such a large number of substitutions in this region cannot be explained solely by the infidelity of Taq DNA polymerase [11], especially since sequence analysis of the other end of the 700-base pair product revealed only 2 out of 203 nucleotide substitutions (one transversion; Fig. 2). Thus, the region of the *X. fastidiosa* genome that surrounds the CVC insert may be especially prone to variation and may prove useful in analysis of the origins of and differences among the host species-specific *X. fastidiosa* strains.

Amplification of DNA directly from bacteria or purified DNA produced more artifactual bands than amplification from infected plant tissue (Fig. 3). Southern hybridization of the cloned CVC-specific RAPD band to PCR products amplified by primers CVC-1 and 272-2 indicated that only the 600-bp product was homologous (data not shown). Despite the appearance of multiple PCR products amplified by the *X. fastidiosa*- or CVC-specific primer pairs, the presence of the specified size product was unambiguous in all cases.

We have developed sets of 20- or 21-base primers that can be used to identify strains of *X. fastidiosa* at the species level (primers 203-1, 203-2; 204-1, 204-2; 208-1, 208-2; 230-1, 230-2; 272-1-int, 272-2-int; Figure 4 top gel). We also developed primers that amplify DNA only from strains that cause CVC (primers CVC-1 and 272-2-int; Figure 4 bottom gel). Previ-

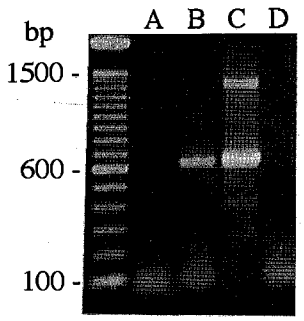

Fig. 3. Amplification of *X. fastidiosa* DNA from CVC-infected citrus. First lane = 100 bp ladder; lane A = noninfected citrus xylem sap; lane B = CVC-infected citrus xylem sap; lane C = positive control – 5000 CVC94-2 bacteria from culture; lane D = no-template control.

ously developed *X. fastidiosa*-specific primers differentiate among strains only through RFLP analysis of the PCR product [14]. In addition to being time consuming, such digestion does not differentiate between strains of *X. fastidiosa* that cause Pierce's disease of grapevine and strains that cause CVC. Additionally, our primers differentiate between *X. fastidiosa* that causes CVC and *X. fastidiosa* associated with citrus blight, which is already present in the United States [12]. The development of a single-step PCR protocol to test potentially CVC-infected citrus budwood is a significant step in preventing the spread of the disease further in Brazil and to countries where it is not endemic. The assay could also be used for epidemiological studies to identify alternative hosts for the pathogen and potential insect vectors.

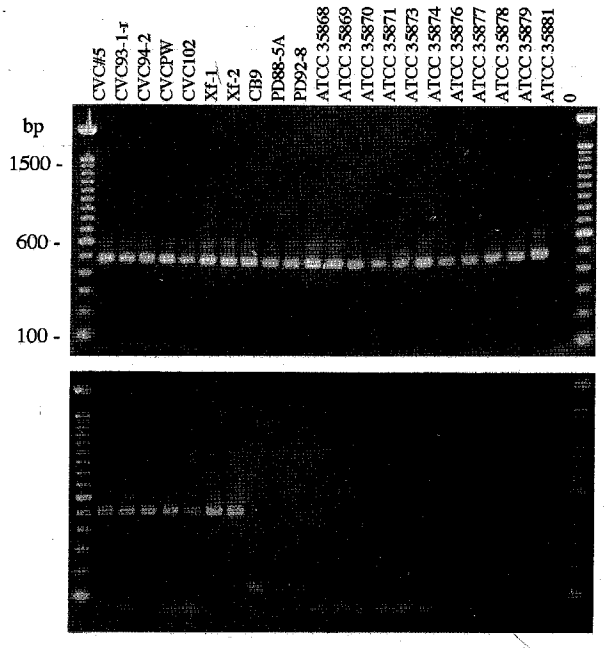

Fig. 4. Amplification of *X. fastidiosa* DNA from intact bacteria with the *X. fastidiosa*-specific 21-base primers 272-1-int and 272-2-int (top gel) or CVC-specific primers CVC-1 and 272-2-int (bottom gel). The outside lanes contain 100-bp marker DNA. The first seven lanes are CVC strains, the next three lanes were isolated by D. Hopkins, the next eleven lanes are ATCC strains (see Table 1 for host), and the last lane contains the no-template control. Approximately 5000 bacteria were used in each 25-μl reaction. Note that the *X. fastidiosa* isolated in Florida from trees with citrus blight (strain CB9) is not a CVC strain.

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