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Amplification of DNA of *Xanthomonas axonopodis* pv. citri from historic citrus canker herbarium specimens

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Abstract

Herbaria are important resources for the study of the origins and dispersal of plant pathogens, particularly bacterial plant pathogens that incite local lesions in which large numbers of pathogen genomes are concentrated. *Xanthomonas axonopodis* pv. citri (*Xac*), the causal agent of citrus bacterial canker disease, is a notable example of such a pathogen. The appearance of novel strains of the pathogen in Florida and elsewhere make it increasingly important to understand the relationships among strains of this pathogen. USDA-ARS at Beltsville, Maryland maintains approximately 700 herbarium specimens with citrus canker disease lesions up to 90 years old, originally collected from all over the world, and so is an important resource for phytogeographic studies of this bacterium. Unfortunately, DNA in herbarium specimens is degraded and may contain high levels of inhibitors of PCR. In this study, we compared a total of 23 DNA isolation techniques in combination with 31 novel primer pairs in order to develop an efficient protocol for the analysis of *Xac* DNA in herbarium specimens. We identified the most reliable extraction method, identified in terms of successful amplification by our panel of 31 primer pairs. We also identified the most robust primer pairs, identified as successful in the largest number of extracts prepared by different methods. We amplified *Xac* genomic sequences up to 542 bp long from herbarium samples up to 89 years old. Primers varied in effectiveness, with some primer pairs amplifying *Xac* DNA from a 1/10,000 dilution of extract from a single lesion from a citrus canker herbarium specimen. Our methodology will be useful to identify pathogens and perform molecular analyses of bacterial and possibly fungal genomes from herbarium specimens.
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Keywords: DNA extraction; DNA purification; Ancient DNA

1. Introduction

The importance of archival and herbarium materials in understanding epidemics of plant diseases has been documented (Ristaino, 1998). By amplification and sequencing of DNA fragments, Ristaino et al.,

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2001 confirmed the identity of *Phytophthora infestans*, the causal agent of potato late blight, in 28 historic herbarium samples, including samples collected between 1845 and 1847 in Ireland and Great Britain (Ristaino et al., 2001).

Citrus canker may have originated in Southeast Asia or India based on symptoms on herbarium specimens from India (1827–1831) and Java (1842–1844) at the Kew Herbarium, England (Fawcett and Jenkins, 1933). The disease is caused by the bacterial pathogen *Xanthomonas axonopodis* pv. citri *(Xac)* (Vauterin et al., 1995) (syn. *Xanthomonas campestris pv. citri*) (Dye et al., 1980). The first recorded outbreak of the disease in Florida occurred in 1913, and the pathogen is believed to have been introduced with infected trifoliate orange (*Poncirus trifoliata*) trees imported from Japan in 1910 for evaluation as citrus rootstocks (Dopson, 1964). This outbreak was contained and subsequently eradicated by 1927 from Florida and from the rest of the U.S. Gulf Coast by 1943 (Dopson, 1964). The disease was not reported subsequently in Florida until 1986 (Schoulties et al., 1987; Hartung and Civerolo, 1987). In spite of eradication efforts, the strain identified in 1986, as well as several different strains of *Xac* have been reported repeatedly, with each report triggering renewed attempts at eradication (Graham et al., 2004). In addition, recent identifications of several new forms of the pathogen in Saudi Arabia (Verniere et al., 1998), and Iran (Khodakaramian and Swings, 2002) in addition to Florida (Cubero and Graham, 2002; Sun et al., 2004) make it increasingly important to understand the diversity and phytogeography of this species. Contemporary work on this topic has been done with strains of the pathogen that were isolated after 1975. Nothing is known about the historic diversity of strains of the bacterium that existed prior to that time.

The Systematic Botany and Mycology laboratory (SBML) of USDA-ARS at Beltsville, MD maintains more than 700 citrus herbarium specimens with clear symptoms of citrus canker, including specimens from the first U.S. outbreak, some collected as early as 1914 (Fawcett and Jenkins, 1933). The collection, which is part of the U.S. National Fungus Collections (BPI), also includes numerous specimens from the Imperial Chinese and Imperial Japanese Herbarium collections, as well as specimens collected in the Philippines, all of which predate World War II. This herbarium collection is a potentially important source of materials for phytogeographic studies of *Xac*. Mavrodieva et al., 2004 detected *Xac* DNA in a limited number of citrus canker herbarium samples collected during the first Florida citrus canker outbreak (Mavrodieva et al., 2004). Their work focused on the development of a real-time polymerase chain reaction (PCR)-based assay for *Xac*, based on a previously identified gene involved in pathogenicity, *pth*A (Swarup et al., 1992). They also confirmed the identity of the pathogen in historic Florida specimens.

There are technical problems that make the study of DNA from very old biological specimens difficult. Chief among these is that such DNA is often severely degraded (Golenberg et al., 1996; Hoss et al., 1996; Paabo, 1985). This precludes the isolation of DNA fragments longer than about 150–400 bp (Golenberg et al., 1996). PCR techniques can solve this problem to various degrees, and also allow the analysis of minute amounts of DNA (Paabo, 1990), thus opening museum collections and herbaria to investigation (Wolfe and Liston, 1998; Lookerman and Jansen, 1996). However, successful amplification and analysis depends not only on the relative preservation of the DNA as discussed above, but also on the methods used to extract and purify the DNA (Hanni et al., 1995). Clearly, the problem of DNA extraction seems crucial for further analyses of herbarium specimens.

The objectives of this study were to develop and validate a simple and robust protocol for the extraction of bacterial DNA from herbarium specimens as well as to develop and validate a suite of PCR primers suitable for the amplification and analysis of *Xac* DNA. To evaluate the suitability of the DNA extracts for amplification, we designed 33 primers used in 31 primer pairs specific for *Xac* by using the whole genome sequence data of *Xac* strain 306 (da Silva et al., 2002). By systematically using DNA extracts prepared by 23 extraction methods for amplification with 31 primer sets, we were able to identify both the best DNA extraction method and the most robust primer combinations for characterization of *Xac* in citrus canker herbarium specimens. We demonstrated the usefulness of the method on herbarium samples up to 90 years old.

2. Materials and methods

2.1. Contemporary plant materials

Contemporary herbarium samples were prepared and used in experiments for the comparison of different DNA extraction methods (Table 1). These specimens were grapefruit (*Citrus paradisi* Macfadyen. Hook.) leaves collected on August 14, 2002, from trees naturally showing symptoms of citrus canker in Pompano Beach, Florida. Nine samples, each containing more than 10 leaves with typical lesions of citrus canker, were pressed between paper and dried in a laboratory oven at ~50 °C for 1 week. These contemporary herbarium specimens were stored in plastic bags at room temperature for two years prior to use.

2.2. Historic herbarium specimens

Twenty citrus specimens with symptoms of citrus canker from the U.S. National Fungus Collection (BPI) (Holmgren et al., 1990) of the USDA-ARS in Beltsville, Maryland were subsequently used to validate our method for the identification of historic *Xac*

Table 1
Comparison of DNA extraction methods and primer combinations for amplification by PCR of *Xanthomonas axonopodis* pv. citri from contemporary citrus canker infected herbarium samples

DNA			PCR amplification results with different primer pairs[a]																																
Extraction method[b]	Yield[c] µg/g	Purity[d]	100 bp amplicons[e]											200 bp amplicons												300 bp amplicons										Total[f]
			P1	P5	A1	A5	B1	B5	C1	C5	D1	D5		P2	P6	A2	A6	B2	B6	C2	C6	D2	D6		P3	P7	A3	A7	B7	C3	C7	D3	D7		
S1H1P1	780.0	1.62	1	1	1	1	1	1	1	1	1	1		1	1	1	1	0	1	1	1	1	1		1	1	1	1	1	1	1	1	1		28
S1H1P4	660.0	1.75	1	1	1	1	1	1	0	1	1	1		1	1	1	1	0	0	1	1	1	1		1	1	1	1	1	1	1	1	1		26
S1H1P2	89.3	1.22	1	0	1	1	1	1	0	1	0	0		0	1	1	0	0	1	1	0	1	1		1	0	1	1	1	1	1	1	1		20
F1H1P1	363.0	1.12	0	1	0	1	1	0	0	1	0	1		1	0	1	0	0	1	0	0	1	1		0	1	1	1	1	0	0	1	0		15
F2H1P1	59.4	1.10	1	0	0	1	1	0	0	1	1	0		1	0	0	0	0	1	1	0	1	1		0	0	1	1	1	1	0	1	0		15
C1H1P1	69.3	1.40	1	0	1	0	1	1	0	1	1	0		0	1	1	0	0	1	1	0	1	0		1	0	1	0	1	0	0	0	0		15
Q2H1P1	145.2	1.95	1	0	1	0	1	1	0	1	1	1		0	1	1	0	0	1	0	0	1	0		1	0	1	0	1	0	0	0	0		14
S1H1P3	534.6	1.10	0	1	0	1	0	1	0	1	0	0		1	0	1	0	0	1	0	0	1	1		0	0	0	0	1	0	0	0	0		10
S1H1P5	554.6	1.23	0	1	0	0	0	1	0	1	0	0		0	1	0	0	0	1	0	0	1	1		0	0	0	0	1	0	0	0	0		8
S1H1P6	N/A	N/A	0	1	0	0	1	0	0	1	0	0		0	0	1	0	0	0	0	0	1	0		0	0	0	0	0	0	0	0	0		5
T1H1P1	386.1	1.35	0	0	0	1	0	0	0	1	0	1		0	0	1	0	0	0	0	0	0	0		0	0	0	1	0	0	0	0	0		5
M4H1P1	643.5	1.08	0	0	0	1	0	0	0	1	0	0		0	0	0	0	0	0	0	0	0	1		0	0	0	0	0	0	0	0	1		4
P1H1P1	402.3	1.10	0	0	0	0	0	0	0	1	0	0		0	0	1	0	0	0	0	0	1	1		0	0	0	0	0	0	0	0	0		4
M2H1P1	297.0	1.29	0	0	0	1	0	0	0	1	0	0		0	0	1	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0		3
M3H1P1	600.6	1.15	0	0	0	1	0	0	0	1	0	0		0	0	0	0	0	0	0	0	0	1		0	0	0	0	0	0	0	0	0		3
M5H1P1	541.2	1.22	0	0	0	0	0	0	0	1	0	0		0	0	0	0	0	0	0	0	0	1		0	0	0	0	0	0	0	0	1		3
M1H1P1	336.6	1.15	0	0	0	0	0	0	0	1	0	1		0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0		2
Q1H1P1	343.2	1.05	0	0	0	0	0	0	0	1	0	0		0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0		1
M3H0P0	N/A	N/A	0	0	0	0	0	0	0	1	0	0		0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0		1
M4H0P0	N/A	N/A	0	0	0	0	0	0	0	1	0	0		0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0		1
M5H0P0	N/A	N/A	0	0	0	0	0	0	0	1	0	0		0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0		1
M1H0P0	N/A	N/A	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0		0
M2H0P0	N/A	N/A	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0		0
Subtotal[g]			6	6	5	11	8	7	1	21	5	6		5	6	12	2	0	8	5	2	11	12		5	3	9	5	7	4	3	7	6		188
Total			76											63											49											188

[a] Successful and failed amplification reactions denoted by '1' and '0' respectively. Extraction methods are listed in descending rank order of extract quality, as judged by the proportion of successful PCR amplifications obtained with each extract.

[b] See Materials and methods. The codes denote the extraction buffers, the homogenization methods, and the purification methods used to prepare the template DNA from contemporary (2 years-old) herbarium samples.

[c] Based on Absorbance at 260 nm.

[d] $A_{260\ nm}/A_{280\ nm}$.

[e] The amplicons are approximately 100, 200 or 300 bp in length.

[f] Summed horizontally for comparison of the quality of the DNA extracts.

[g] Summed vertically for comparison of the robustness of the primer pairs.

Table 2
Primers used to amplify genomic and plasmid DNA of *Xanthomonas axonopodis* pv. citri from citrus canker herbarium specimens

Primer name	Sequence 5′ - 3′	Pairs with	Pair name	Consensus Tm	Anneals to gene[a]	Amplicon size (bp)
A0033d	AACGTGGGGATACCTAAG	N/A	N/A	51	Multiple genes	N/A
A0033u	AAGAAATGTGGGGTTTCC	N/A	N/A	50	Multiple genes	N/A
A0032a	AAGCCAAGAATTTCGGCG	A0033d	P1	51	XACa0032-0033	115
A0032b	CATCCGCATCTTCGAGTA	A0033d	P2	51	XACa0032-0033	210
A0032c	CAAATTCTTCGCTGCCGA	A0033d	P3	51	XACa0032-0033	306
A0034a	ACTTTCTCGAAGGAGTCG	A0033u	P5	50	XACa0033-0034	106
A0034b	CGTTGACCAAACCGAATG	A0033u	P6	50	XACa0033-0034	205
A0034c	GTGAAGCCAGAAGACGAG	A0033u	P7	50	XACa0033-0034	317
0092a	TAATTCCTTCCTCGCGTC	A0033d	A1	51	XAC0092-0093	121
0092b	AAGCG CGTTGCATACCA	A0033d	A2	51	XAC0092-0093	202
0092c	TACGTAACCTTGAAGCAC	A0033d	A3	51	XAC0092-0093	325
0092d	CTGTAGTTTCTCTCTGCC	A0033d	A9	51	XAC0092-0093	542
0092e	ACTGCACTTCCAAATCCG	A0033d	A10	51	XAC0092-0093	708
0094a	AAGCGATGAAGCGGCTG	A0033u	A5	51	XAC0093-0094	110
0094b	CAATGAAGCGCAACGTCA	A0033u	A6	51	XAC0093-0094	224
0094c	CTTCAACTGCGAGCAATA	A0033u	A7	51	XAC0093-0094	302
0501a	TCTGAACACGCACCGCA	A0033d	B1	52	XAC0501-0502	109
0501b	CGCGCTGTCCAGTGATA	A0033d	B2	52	XAC0501-0502	190
0503a	ATCGTCACGCAGCACATC	A0033u	B5	52	XAC0501-0502	102
0503b	CATCCAGCAACAGCGGC	A0033u	B6	52	XAC0502-0503	205
0503c	GCAGATCGATCCGCTGC	A0033u	B7	52	XAC0502-0503	325
1928a	TAAGGGTTGAGGTTGCGC	A0033d	C1	51	XAC1928-1929	103
1928b	GCTAGCGGTAGAAGGGA	A0033d	C2	51	XAC1928-1929	212
1928c	CTGATGCCAAGAGACTG	A0033d	C3	51	XAC1928-1929	348
1930a	ATCGCAGCCGATCTATG	A0033u	C5	50	XAC1929-1930	108
1930b	GATAATCGGGAATCGGGA	A0033u	C6	50	XAC1929-1930	209
1930c	CGCTGATCCTGGATGTG	A0033u	C7	50	XAC1929-1930	301
4324a	TCTGAGTGAACTTGACCC	A0033d	D1	50	XAC4324-4325	107
4324b	TAGGCAAGCGACACTTTG	A0033d	D2	50	XAC4324-4325	203
4324c	CTTGATCAGGCGATACTC	A0033d	D3	50	XAC4324-4325	315
4326a	GTGGTGTGTACCTGTGG	A0033u	D5	51	XAC4325-4326	110
4326b	CAGAGGCGCTCGATTAC	A0033u	D6	51	XAC4325-4326	214
4326c	GTGCCTTACGCGTGATG	A0033u	D7	51	XAC4325-4326	369

[a] Based on full genome sequence (da Silva et al., 2002).

DNA by PCR using the best DNA extraction method and most robust primer combinations (Table 2).

2.3. Sampling

Xac is not a systemic pathogen, causing clearly delimited lesions on leaves, stems and fruits, in which the pathogen is highly concentrated. Lesions were cut just outside their boundary with a razor blade to an area of about 25 mm^2. For comparison of DNA extraction methods, 10 lesions, adjusted to 30 mg by trimming boundary areas, were used for each sample. To estimate the sensitivity of the best DNA extraction method, extracts were prepared from 1 – 10 lesions from the contemporary specimens. In experiments with historic herbarium samples, extracts were prepared from 10 mg dry weight of leaf, fruit or stem lesions. To avoid cross contamination, each herbarium sample was opened, cut and weighed by using a new razor blade, new examination gloves and new weighing paper in a room where no bacteriological work had been conducted previously and no PCR experiments had ever been performed.

2.4. Extraction of total DNA from herbarium samples

Twenty-three DNA extraction methods were developed by altering three factors: extraction solutions,

tissue homogenization, and purification methods. The treatments are summarized and denoted in Table 1 using a 6 member alphanumeric code. The first two digits of the code denote the extraction solution, the second two digits denote homogenization, and the last two digits denote the purification method.

(i) The following 13 extraction solutions were compared. S1, Sorbitol/CTAB buffer (Storchova et al., 2000; Ristaino et al., 2001); T1, TE buffer, (0.2 M Tris, 0.05 M EDTA, pH7.5) containing 2% 2-mercaptoethanol; C1, CTAB buffer (Witzell, 1999); Q1, Qiagen buffer AL from QIAamp DNA Blood Mini kit; Q2, Qiagen buffer AP1 from DNeasy Plant Mini kit (Qiagen, Valencia, CA); F1, 480 μl FastDNA Buffer CLS-VF from the FastDNA kit (Qbiogene, Carlsbad, CA.) and 120 μl PBS; F2, 480 μl of buffer CLS-TC from the FastDNA kit (Qbiogene, Carlsbad, CA.) and 120 μl PBS; P1, PBS (pH 7.4) containing 0.5% PVP-40, and 1% β-mercaptoethanol. In addition, the following solutions were used for extractions: (Mavrodieva et al., 2004): M1, Sterile Water; M2, Saturated $CaCO_3$ in sterile water; M3, Silwet L-77 in water 1 : 5000; M4, Silwet L-77 in saturated $CaCO_3$ in water 1 : 5000; M5, 5% Chelex 100 in Silwet L-77 in saturated $CaCO_3$ in water 1 : 5000. The volume of the extraction buffer was set at 600 μl for all samples, except for solutions M1–M5 where only 200 μl was used. Buffers T1, C1, Q1, Q2 and P1 also contained 4 μl of RNAse A (100 mg/ml).

(ii) Samples were either allowed to soak in buffer or were homogenized as follows: All plant samples in this study were put into sterile lysing matrix A tubes (Qbiogene, Carlsbad, CA). Sample extracts were obtained in 200 μl of the buffers M1, M2, M3, M4, and M5, respectively, by simply soaking and shaking (250 rpm) the plant samples in buffers, without homogenization (H0) or subsequent DNA extraction or purification (Mavrodieva et al., 2004). Homogenization (H1) in the other extraction solutions was done at speed setting 4.0 for 20 s with a FastPrep FP120 instrument using a 6.3 mm cylindrical ceramic sphere (Qbiogene, Carlsbad, CA). Cellular lysis was completed by incubating the homogenized mixture for 10 min at 65 °C.

(iii) DNA purification, elution and quantification. Following the homogenization treatments above (H1), samples were centrifuged for 5 min at 14,000 RPM in a microcentrifuge and the supernatants were transferred to new tubes and purified by one of the following six methods (P1–P6). P1, one volume of 100% ethanol was added to the extract, mixed, and incubated for 5 min on ice. The mixture was applied to a QIAamp spin column (Qiagen, Valencia, CA), and the manufacturer's protocol was modified as follows: The extracts were centrifuged at 14,000 RPM for 1 min, and the filter was transferred to a new 1.5 ml tube. The filter was washed two times in 500 μl 70% ethanol by centrifuging at maximum speed for 1 min. The filter was centrifuged for another 1 min at maximum speed to eliminate any residual ethanol and then was transferred to a new 1.5 ml tube, 100 μl TE buffer was added and incubated at room temperature for 5 min followed by centrifugation at 8000 rpm for 1 min to collect the DNA extract. P2, modified CTAB purification (Witzell, 1999). P3, DNA binding matrix (Qbiogene, Carlsbad, CA); P4, DNeasy Plant Kit (Qiagen, Valencia, CA); P5, Wizard Magnetic 96 well Plant DNA (Promega, Madison, WI); P6, Whatman FTA paper (Whatman, Clifton, NJ). The manufacturer's protocols were followed when using the kits described as treatments P3–P6. All DNA extracts were eluted in TE buffer, quantified and stored at −20 °C. DNA yield and purity were estimated by measuring OD_{260nm} and $OD_{260nm/280nm}$, respectively, with a UV-visible recording spectrophotometer Model UV-160 (Shimadzu, Columbia, MD). Extracts obtained by diffusion rather than homogenization (H0) were used directly for PCR without further purification (P0).

2.5. Primer design and in Silico PCR

Primers for amplification of *Xac* genomic DNA (Table 2) were designed using the following criteria: (i) primers should have about 60% GC content based on the 64.7% GC content of the whole genome (da Silva et al., 2002); (ii) primers should have a unique binding site in the *Xac* genome since a unique amplicon was preferred; (iii) the amplification product should span one end of the insertion site of a transposable element since transposable elements may be located near strain-specific genes (da Silva et al., 2002); (iv) amplicons of about 100, 200, 300, and 500 bp were specified so that we could assess the physical degradation of the samples. A primer pair which specified a 708 bp amplicon was used to detect

Table 3
Herbarium specimens with symptoms of citrus canker[a] and amplification results for detection of *Xanthomonas axonopodis* pv. citri

Herbarium specimens					PCR amplicons (bp) obtained with different primer pairs										
BPI #	Year[b]	Origin	Host	Tissues	Yield μg/g	Purity OD260/280	100 bp[c]		200 bp		300		542	708	Total
							A5	C5	A2	D2	A3	D7	A9	A10	
686504	1915	MS, USA	*C. grandis*	Leaves	165.0	1.71	1	1	1	1	0	0	0	0	4
686399	1915	TX, USA	*Citrus sp.*	Leaves	181.5	1.39	1	1	1	1	0	1	1	0	6
686403	1915	TX, USA	*Citrus sp.*	Stems	350.9	1.61	1	1	0	1	1	0	0	0	4
686402	1915	DC, USA	*Citrus sp.*	Leaves	718.0	1.88	1	1	1	1	0	0	0	0	4
686427	1915	AL, USA	*Citrus sp.*	Leaves	768.4	1.57	1	1	1	0	0	0	0	0	3
686404	1915	AL, USA	*Citrus sp.*	Leaves	657.2	1.55	1	1	1	0	0	0	0	0	3
686317	1916	Philippines	*Citrus sp.*	Leaves	685.3	1.75	1	1	1	1	0	1	0	0	5
686320	1916	Philippines	*Citrus sp.*	Leaves	745.8	1.70	1	1	1	1	1	0	0	0	5
686321	1916	Philippines	*Citrus sp.*	Leaves	698.2	1.59	1	1	0	1	0	0	0	0	3
686318	1919	Philippines	*Citrus sp.*	Leaves	745.5	1.45	1	1	1	0	0	0	0	0	1
686352	1920	Thailand	*Citrus sp.*	Stems	736.6	1.65	1	1	1	1	0	1	0	0	5
686353	1920	Thailand	*Citrus sp.*	Leaves	686.4	1.67	1	1	0	1	1	0	0	0	4
686354	1920	Thailand	*Citrus sp.*	Leaves	755.8	1.54	1	1	0	0	0	0	0	0	2
686322	1945	Thailand	*Citrus sp.*	Fruit	746.5	1.41	1	1	0	0	0	0	0	0	2
686316	1949	Thailand	*Citrus sp.*	Fruit	660.7	1.21	1	1	1	1	1	0	0	0	5
686319	1949	Philippines	*Citrus sp.*	Fruit	323.4	1.03	1	1	0	1	0	1	0	0	4
686058	1951	India	*C. mitis*	Fruit	712.5	1.09	1	1	0	0	0	0	0	0	2
686400	1975	Philippines	*Citrus sp.*	Leaves	764.0	1.54	1	1	0	0	0	0	0	0	2
686401	1977	Indonesia	*Citrus sp.*	Leaves	715.3	1.57	1	1	1	1	1	0	0	0	5
	2002	FL, USA	*C. sinensis*	Leaves	767.4	1.62	1	1	1	1	1	1	1	0	7
						Subtotal	20	20	12	13	6	5	2	0	
						Total									76

[a] From herbarium samples maintained by the Systematic Botany and Mycology Laboratory, USDA ARS, Beltsville MD, as part of the U.S. National Fungus Collections (BPI) (Holmgren et al., 1990).
[b] Year the samples were collected and dried.
[c] Amplicons of approximately 100, 200 or 300 bp and exactly 542 and 708 bp were predicted. DNA extraction method S1H1P1 (Table 1) was used to prepare these DNA extracts. See Table 3 for precise amplicon sizes.

contamination with contemporary DNA. In Silico PCRs were performed for all primer pairs before they were synthesized by using BLASTn against the NCBI GenBank database to ensure the specificity of the amplicons.

2.6. PCR amplification

PCR reactions were performed in a final volume of 10 μl containing 200 nm of each primer, 200 mM dNTPs, 2.5 mM $MgCl_2$, 0.5 unit *Taq* polymerase (Roche, Indianapolis, IN) and 1 μl of DNA template. The amplification program began with incubation at 94 °C for 4 min, followed by 45 cycles of denaturation at 94 °C for 30 s, annealing at the consensus Tm of the primer pair −3 °C for 30 s (Table 2), and extension at 72 °C for 1 min, followed by a final extension cycle of 10 min at 72 °C. PCR products were visualized by staining with ethidium bromide following electrophoresis through 1.0% agarose gels.

3. Results

3.1. Total DNA extraction yield

DNA yields ranged from 59.4 to 780.0 μg/g citrus canker lesions (Tables 1 and 3). There were substantial differences in DNA yields obtained with different extraction and purification methods (Table 1). The highest DNA yields were obtained with sorbitol buffer system (S1), followed by $CaCo_3$–Silwet–Chelex buffers M4, M3, and M5. The FastDNA buffer CLS-TC (F2) and the CTAB buffer (C1) yielded the least DNA from the citrus canker herbarium samples. We attribute much of the difference in DNA yield among the

different purification systems to the difficulty of removing the supernatants from the lysates following homogenization and incubation at 65 °C for 10 min. This was very difficult for the CTAB buffer (C1) and the FastDNA buffer CLS-TC (F2), even after the lysates had been centrifuged at maximum speed for 10 min. Although this CTAB buffer system was used for the extraction of DNA from herbarium specimens of other plant species (Ristaino et al., 2001; Drabkova et al., 2002), it was not the best for obtaining DNA from citrus canker herbarium samples. Increasing the lysis and precipitation times for the CTAB method did not improve its DNA yields (data not shown).

3.2. Purity and amplification quality of extracts

We compared seven DNA purification systems following homogenization (P0–P6, Table 1) in sorbitol buffer (S1), with a FastPrep 120 instrument. In terms of purity estimated by the $OD_{260/280}$, the DNeasy Plant kit (S1H1P4) was the best, closely followed by the simple column (S1H1P1). There were no obvious differences in DNA purity among the CTAB method (S1H1P2), Wizard magnetic system (S1H1P5) and DNA binding matrix (S1H1P3).

To evaluate the amplification quality of the DNA extracts, we designed 33 primers used in 31 primer pairs based on the whole genome sequence of *Xac* strain 306 (da Silva et al., 2002) (Table 2). Twenty-nine primer pairs were used to create amplicons of approximately 100 – 300 bp, and we amplified these relatively short targets with high specificity and efficiency. Our amplification of *Xac* DNA from historic citrus canker herbarium samples was carried out in a single round of PCR, and there was no occurrence of false positives in this study. There was also no evidence of contamination with contemporary DNA, since primer pair A10, predicted to produce an amplicon of 708 bp (Table 3), did not produce amplicons from any herbarium samples.

When extracts of *Xac* from citrus canker herbarium samples obtained with sorbitol extraction buffer by the simple column purification method were used for PCR, 28/29 (97%) of the primer pairs tested yielded amplicons of the expected size (S1H1P1; Table 1). The DNA extract obtained with the DNeasy Plant kit (S1H1P4) produced specific bands with 26 of the 29 (90%) primer pairs, and the DNA extract obtained with the CTAB extraction modified with sorbitol extraction buffer (S1H1P2) produced amplicons with 20 of the 29 (69%) primer pairs, in spite of its relatively low yield. Purification by other methods was much less satisfactory, with 14/23 methods producing DNA that could be amplified in 20% or less of the reactions (Table 1). Protocol S1H1P1 produced extracts that were excellent in terms of DNA yield, purity, amplification quality (Table 1) and sensitivity (Table 4). With this protocol, DNA yields were well correlated with the weight of lesion samples used, and 1.5 μg DNA could be extracted from a single dried lesion (2.0 mg) (Table 4).

3.3. Amplicon size

We used 10, 10, and 9 primer pairs to amplify PCR product sizes of approximately 100, 200, and 300 bp,

Table 4
Sensitivity of the DNA extraction method[a]

Number of leaf lesions	Weight of lesions mg	DNA yield μg	DNA purity O.D260/O.D.280	PCR results with primer combinations[b]			
				A2	B1	C3	D3
				202	109	348	315
10	27.1	12.55	1.5688	+	+	+	+
8	16.9	10.75	1.5000	+	+	+	+
6	12.1	8.16	1.6000	+	+	+	+
4	8.6	6.28	1.6719	+	+	–	+
2	4.0	3.25	1.7174	+	+	–	–
1	2.0	1.50	1.6190	+	–	–	–

[a] Method 'S1H1P1'. Extraction buffer containing sorbitol; Sample homogenized with the Fastprep instrument; extract purified using a QIAmp filter procedure with modifications. See the Materials and methods for details.
[b] Primer pair designation and amplicon size in base pairs (Table 2).

respectively, from DNA extracts obtained by different extraction methods from the 2-year-old citrus herbarium samples with lesions of citrus canker (Table 1). In terms of size, among the 188 amplicons produced by the 29 primer pairs in this initial portion of our study (Table 1), 76 (40%) were about 100 bp, 63 (34%) about 200 bp, and 49 (26%) about 300 bp.

A subset of 8 primer pairs were used to amplify DNA from the historic herbarium specimens. Among the 76 amplicons obtained from these DNA extracts, 40 (52.63%) were about 100 bp, 25 (32.89%) about 200 bp, and 11 (14.47%) about 300 bp (Table 2). In terms of successful amplification of targets from historic extracts, PCR products approximately 100 bp in length were amplified from all 20 (100%) of the herbarium specimens. Fifteen samples (75%) yielded the expected products of approximately 200 bp in size. Nine samples (45%) yielded products of approximately 300 bp. Only two samples (2.63%) yielded products of 542 bp. As expected, no amplicons of 708 bp were obtained with primer pair A10 (Table 3) from any of 20 herbarium specimens. This primer was included in the experiments as a control for potential contamination of samples with contemporary *Xac* DNA from the laboratory.

4. Discussion

We wanted to develop and validate a simple, robust method to isolate bacterial pathogen DNA from herbarium specimens. We tested both popular kits and previously published procedures designed to extract DNA from samples and then characterized the extracts for DNA quantity, purity and the presence of inhibitors of PCR. Because we expected the DNA to be degraded, we also designed a set of 33 primers that could be used to direct the amplification of products that ranged in size from 102 to 708 bp. By using our set of primers with our set of DNA extracts, we have identified the best DNA extraction and purification methods. These were identified because extracts produced by these methods supported amplification by the greatest number of PCR primer pairs. We also have identified the most robust primer pairs for use with these extracts. These were identified as being able to produce amplification products from the largest number of extracts, which varied in quality (Table 1). All amplification products in this study were cloned and sequenced and verified to be of *Xac* origin by comparison with the published genomic sequence of this bacterium (da Silva et al., 2002). The analysis of this sequence data is the subject of a future report (Li et al., in preparation).

The results of our analyses of contemporary and historic herbarium specimens show that the *Xac* DNA in herbarium specimens was degraded into small fragments, primarily during the drying process itself, rather than over an extended period of time. Our yields of total DNA, as high as 780 ug/g tissue, compared favorably with those of other workers starting with dried plant material ground in a mortar and pestle. The yield of DNA obtained from herbarium specimens of Juncaceae ranged from 1.3 to 45.5 μg/g dried leaves (Drabkova et al., 2002). The yield of DNA ranged from 164 to 494 μg/g from dried foliar samples of market teas using a modified CTAB procedure (Singh et al., 1999). The excellent DNA yields in the current work indicate that our FastPrep FP120 instrument with ceramic spheres is effective for extraction of DNA from herbarium specimens. When labor savings and the reduced potential for cross contamination of samples are considered, this instrument is an excellent option for this purpose. A mixer mill (Qiagen, Chatsworth, CA) could also be a good alternative for this purpose (Drabkova et al., 2002).

The DNA extraction and purification methods described here could be very useful for diagnosis of other bacterial and fungal organisms in citrus herbarium and quarantine samples, and for DNA-based research on historic DNA of other herbarium specimens. We will use our best extraction method and primers based on the full genome sequence of the pathogen to characterize the diversity of *Xac* in historic herbarium specimens. Because the herbarium specimens were collected over a long period of time worldwide, we will have a unique opportunity to characterize the diversity present in *Xac* today as compared to in the past. The 31 primer pairs developed in this study also will be useful for the identification of the pathogen in contemporary samples. The data in Table 1 can be used to directly estimate the robustness of each primer pair for this purpose. Primer pair C5, with an amplification product of 108 bp (Table 2), may be especially well suited for this purpose. Finally, we note that our work would not have been possible if earlier researchers had not

deposited voucher specimens from their research in herbaria. Future researchers will benefit if contemporary researchers also contribute voucher specimens from their work to herbaria.

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References

Cubero, J., Graham, J.H., 2002. Genetic relationship among worldwide strains of *Xanthomonas* causing canker in citrus species and design of new primers for their identification by PCR. Applied and Environmental Microbiology 68, 1257–1264.

da Silva, A.C.R., Ferro, J.A., Reinach, F.C., Farah, C.S., Furlan, L.R., Quaggio, R.B., Monteiro-Vitorello, C.B., Van Sluys, M.A., Almeida, N.F., Alves, L.M.C., do Amaral, A.M., Bertolinni, M.C., Camargo, I.E.A., Camarotte, G., Cannavan, F., Cardozo, J., Chambergo, F., Ciapina, L.P., Cicarelli, R.M.B., Coutinho, L.L., Cursino-Santos, J.R., El-Dorry, H., Faria, J.B., Ferreira, A.J.S., Ferreira, R.C.C., Ferro, M.I.T., Formighieri, E.F., Franco, M.C., Greggio, C.C., Gruber, A., Katsuyama, A.M., Kishi, L.T., Leite, R.P., Lemos, E.G.M., Lemos, M.V.F., Locall, E.C., Machado, M.A., Madeira, A.M.B.N., Marinez-Rossi, N.M., Martins, E.C., Meidanis, J., Menck, C.F.M., Miyaki, C.Y., Moon, D.H., Moreira, L.M., Novo, M.T.M., Okura, V.K., Oliveira, M.C., Oliveira, V.R., Pereira, H.A., Rossi, A., Sena, J.A.D., Silva, C., de Souza, R.F., Spinola, R.A.F., Takita, M.A., Tamura, R.E., Teixera, E.C., Tezza, R.I.D., Trinidade dos Dantos, M., Truffi, D., Tsai, S.M., White, F.F., Setubal, J.C., Kitajima, J.P., 2002. Comparison of the genomes of two *Xanthomonas* pathogens with differing host specificities. Nature 417, 459–463.

Dopson, R.N., 1964. The eradication of citrus canker. Plant Disease Reporter 48, 30–31.

Drabkova, L., Kirschner, J., Vlcek, C., 2002. Comparison of seven DNA extraction and amplification protocols in historical herbarium specimens of *Juncaceae*. Plant Molecular Biology Reporter 20, 161–175.

Dye, D.W., Bradbury, J.F., Goto, M., Hayward, A.C., Lelliot, R.A., Schroth, M.N., 1980. International standards for naming pathovars of phytopathogenic bacteria and a list of pathovar names and pathotype strains. Review of Plant Pathology 59, 153–168.

Fawcett, H.S., Jenkins, A.E., 1933. Records of citrus canker from herbarium specimens of the genus Citrus in England and the United States. Phytopathology 23, 820–824.

Golenberg, E.M., Bickel, A., Weihs, P., 1996. Effect of highly fragmented DNA on PCR. Nucleic Acids Research 24, 5026–5033.

Graham, J.H., Gottwald, T.R., Cubero, J., Achor, D.S., 2004. *Xanthomonas axonopodis* pv. *citri*: factors affecting successful eradication of citrus canker. Molecular Plant Pathology 5, 1–15.

Hanni, C., Brousseau, T., Laudet, V., Stehelin, D., 1995. Isopropanol precipitation removes PCR inhibitors from ancient bone extracts. Nucleic Acids Research 23, 881–882.

Hartung, J.S., Civerolo, E.L., 1987. Genomic fingerprints of *Xanthomonas campestris* pv. *citri* strains from Asia, South America and Florida. Phytopathology 77, 282–285.

Holmgren, P.K., Holmgren, N.H., Barnett, L.C., 1990. Index Herbariorum: Part I. The herbaria of the world, 8th edition. Regnum Vegetabile, vol. 120. 630 pp.

Hoss, M., Jayuga, M., Dizdaroglu, M., Paabo, S., 1996. DNA damage and DNA sequence retrieval from ancient tissues. Nucleic Acids Research 24, 2857–2867.

Khodakaramian, G., Swings, J., 2002. AFLP fingerprinting of the strains of *Xanthomonas axonopodis* inducing citrus canker disease in southern Iran. Journal of Phytopathology 150, 227–231.

Lookerman, D.J., Jansen, R.L., 1996. The use of herbarium materials for DNA studies. In: Steussy, T.F., Sohmer, S.H. (Eds.), Sampling the Green World. Columbia University Press, New York, pp. 205–220.

Mavrodieva, V., Levy, L., Gabriel, D.W., 2004. Improved sampling methods for real-time polymerase chain reaction diagnosis of citrus canker from field samples. Phytopathology 94, 61–68.

Paabo, S., 1985. Molecular cloning of ancient Egyptian mummy DNA. Nature 314, 644–645.

Paabo, S., 1990. Amplifying ancient DNA. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols: A Guide to Methods and Applications. Academic Press, San Diego, CA, pp. 159–166.

Ristaino, J.B., 1998. The importance of archival and herbarium materials in understanding the role of oospores in late blight epidemics of the past. Phytopathology 88, 1120–1130.

Ristaino, J.B., Groves, C.T., Parra, G.R., 2001. PCR amplification of the Irish potato famine pathogen from historic specimens. Nature 411, 695–697.

Schoulties, C.L., Civerolo, E.L., Miller, J.W., Stall, R.E., Krass, C.J., Poe, S.R., 1987. Citrus canker in Florida. Plant Disease 71, 388–395.

Singh, M., Bandana, Ahuja, P.S., 1999. Isolation and PCR amplification of genomic DNA from market samples of dry tea. Plant Molecular Biology Reporter 17, 171–178.

Storchova, H., Hrdliekova, R., Chrtek Jr., R., Tetera, M., Fritze, D., Fehrer, J., 2000. An improved method of DNA isolation from plants collected in the field and conserved in saturated NaCL/CTAB solution. Taxon 49, 79–84.

Sun, X.A., Stall, R.E., Jones, R.E., Cubero, J., Gottwald, T.R., Graham, J.H., Dixon, W.N., Schubert, T.S., Chaloux, P.H., Stromberg, V.K., Lacy, G.H., Sutton, B.D., 2004. Detection and characterization of a new strain of citrus canker bacteria from Key/Mexican lime and alemow in South Florida. Plant Disease 88, 1179–1188.

Swarup, S., Yang, Y., Kingsley, M.T., Gabriel, D.W., 1992. An *Xanthomonas citri* pathogenicity locus, *pthA*, pleitrophically encodes gratuitous avirulence on nonhosts. Molecular Plant-Microbe Interactions 5, 204–213.

Vauterin, L., Hoste, B., Kersters, K., Swings, J., 1995. Reclassification of *Xanthomonas*. International Journal of Systematic Bacteriology 45, 472–489.

Verniere, C., Hartung, J.S., Pruvost, O.P., Civerolo, E.L., Alvarez, A.M., Maestri, P., Luisetti, J., 1998. Characterization of phenotypically distinct strains of *Xanthomonas axonopodis* pv. *citri* from Southwest Asia. European Journal of Plant Pathology 104, 477–485.

Witzell, H., 1999. Chloroplast DNA variation and reticulate evolution in sexual and apomictic sections of dandelions. Molecular Ecology 8, 2023–2035.

Wolfe, A.D., Liston, A., 1998. Contribution of PCR-based methods to plant systematics and evolutionary biology. In: Soltis, D.E., Soltis, P.S., Doyles, J.J. (Eds.), Molecular Systematics of Plants. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 43–86.