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Specific detection of *Phytophthora cactorum* in diseased strawberry plants using nested polymerase chain reaction

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Validated protocols for DNA purification and PCR amplification are reported for detection of *Phytophthora cactorum* in diseased strawberry plants. To remove PCR inhibitors, necrotic strawberry tissues were soaked in 5% alconox solution for >12 h before DNA extraction, and the extracted genomic DNA was embedded in an agarose gel chamber and subjected to electrophoresis. The purified DNA was amplified reliably by PCR. Nested PCR was used to detect a portion of the rRNA gene of *P. cactorum* in samples. In the first round of PCR, primers ITS1 and ITS4 amplified fragments of varying sizes from total genomic DNA from diseased strawberry plants. In the second round of PCR, a 1:25 dilution of the first-round PCR products was used as template with two *P. cactorum*-specific primer pairs (BPhycacL87FRG and BPhycacR87RRG, which amplified a 340-bp fragment and a 480-bp fragment from the rRNA gene; and BPhycacL89FRG and BPhycacR176RRG, which amplified a 431-bp fragment). Validation tests using culture-based isolations as a standard for comparison indicated that the DNA purification and PCR primers and amplification protocols were reliable and specifically amplified a portion of the rRNA gene of *P. cactorum* from necrotic root, crown and petiole tissues of strawberry naturally infected by the pathogen.

Keywords: CTAB method, detection, diagnosis, *Fragaria,* optimization, soilborne pathogens

Introduction

Phytophthora cactorum can infect the roots, crowns and fruits of strawberry plants and is among the most serious soilborne pathogens affecting the crop worldwide (Maas, 1998; Strand, 1994). In California, *P. cactorum* has caused serious yield losses through plant stunting and collapse, especially on susceptible cultivars such as Diamante and Ventana (G. T. Browne & R. G. Bhat, unpublished data). Although it often causes extensive necrosis in infected plant parts, *P. cactorum* can be carried on or in planting stock without obvious symptoms of disease (G. T. Browne & R. G. Bhat, unpublished data). Strawberry nurserymen use diagnostic services in their efforts to insure that their stock is free from pathogens, while strawberry fruit growers rely on diagnostics to discriminate among causes of soilborne diseases with similar symptoms so that appropriate management steps can be taken promptly. Symptoms of disease caused by *P. cactorum* can be confused with those caused by *P. fragariae* var. *fragariae, P. citricola, Colletotrichum acutatum* and *Verticillium dahliae*, which also are important causes of strawberry plant collapse in California and worldwide.

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Culture-based isolation approaches are still widely used for detection of *P. cactorum* in strawberry, but, with appropriate optimization and validations, PCR may offer important advantages over culturing and other approaches available for monitoring this pathogen. Culture-based detection of *Phytophthora* species requires approximately 1 week and specialized mycological skills, whereas PCR-based detection, including DNA extraction, generally requires 2–3 days and general training in molecular biology. Enzyme-linked immunological tests are available for detection of *Phytophthora* spp., but they are not reliably species-specific (Amouzou-Alladaye *et al.*, 1988; MacDonald *et al.*, 1990; Olsson, 1995). Recently, an antibody-based lateral flow device (LFD) was validated for quick *in situ* field detection of *P. kernoviae* and *P. ramorum* in foliage of their hosts (Lane *et al.*, 2007).

Diagnostic PCR methods and primers have been developed for *Phytophthora* species that infect strawberry, including *P. cactorum* (Lacourt *et al.*, 1997; Causin *et al.*, 2005; Schena *et al.*, 2008) and *P. fragariae* var. *fragariae* (Bonants *et al.*, 1997, 2004; Lacourt *et al.*, 1997; Hughes *et al.*, 1998; Ioos *et al.*, 2006). Nested PCR primers ADF1 and ADR1 were reported to amplify a 520-bp DNA fragment from the ITS regions of the rRNA gene of *P. cactorum* in root samples of strawberry (Lacourt *et al.*, 1997). The PCR primers PC1/PC2, designed as SCAR markers from a specific

random amplified polymorphic DNA fragment to amplify a PCR product of approximately 450 bp, were reported to be specific, sensitive and robust for detecting *P. cactorum* in several host plants including strawberry (Causin *et al.*, 2005). Recently, primers Ycac1F and Ycac2R, that amplify a 192-bp portion of the *ras*-related protein gene, *Ypt*1, were reported to be specific for *P. cactorum* (Schena *et al.*, 2008). At the time of this research project, primer pairs PC1/PC2 and Ycac1F/Ycac2R were not available for testing, but primers ADF1 and ADR1 were examined in this study as described below.

Inhibitors of PCR have been an important impediment in development and application of reliable PCR diagnostics for plant disease samples (Wilson, 1997; Watson & Blackwell, 2000; Atkins & Clark, 2004). This was found to be true for diseased tissues from strawberry plants (R. G. Bhat & G. T. Browne, unpublished data).

The overall goal of this research was to develop practical PCR-based protocols for detection of *P. cactorum* in strawberry plants by optimizing DNA extraction and purification procedures and PCR conditions, designing primers with improved specificity, and conducting validations using field samples. A portion of this work was reported previously (Bhat & Browne, 2007).

Materials and methods

Isolates of *Phytophthora* and fungi

DNA was extracted from mycelium of *P. cactorum* (five isolates), 15 other species of *Phytophthora*, *Verticillium dahliae*, *Colletotrichum acutatum* and *Macrophomina phaseolina* for use in developing and evaluating PCR protocols for detection of *P. cactorum* in strawberry (Table 1). The *Phytophthora* spp. were subcultured from single-zoospore colonies and stored at 14°C under sterile mineral oil on V8 juice agar (V8JA). The fungi were subcultured from single-conidial colonies, grown on potato dextrose agar (PDA) medium in vials, and stored on the laboratory bench at 22°C.

DNA extraction and purification

Total genomic DNA was extracted from mycelia of the *Phytophthora* spp. and fungi listed above after growth in

Table 1 Isolates used for testing of PCR primers and protocols

Plant pathogen	Isolate[a]	Host	PCR product[b]	
			Previously reported primers[c]	Primers designed in this study[d]
Phytophthora cactorum	gb1750, gb2490Rts, gb2990a, gb3656, gb4028,	Strawberry	+	+
Phytophthora cambivora	gb3346	Almond	–	–
Phytophthora capsici	gb3300	Tomato	+	–
Phytophthora cinnamomi	gb647	Walnut	–	–
Phytophthora citricola	skTri02-03-07	Strawberry	+	–
Phytophthora citrophthora	sm3477	Citrus	+	–
Phytophthora cryptogea	gb343	Carrot	–	–
Phytophthora drechsleri	gb2065	Almond	+	–
Phytophthora fragariae	P-3295	Raspberry	–	–
Phytophthora infestans	R1-US6	Potato	–	+
Phytophthora lateralis	sm3338	Unknown	+	–
Phytophthora megasperma	gb2658	Strawberry	+	–
Phytophthora nemorosa	P-43	Oak	+	–
Phytophthora parasitica	gb1970	Almond	+	–
Phytophthora ramorum	Pr-104sz	Oak	+	–
Phytophthora syringae	gb2007	Almond	–	–
Verticillium dahliae	MBA10	Strawberry	–	–
Colletotrichum acutatum	CacuSy	Strawberry	–	–
Macrophomina phaseolina	Mpha01	Strawberry	NT	–

[a]Letters 'gb' and 'sm' indicate isolates from G. T. Browne and S. M. Mircetich, respectively, USDA-ARS, University of California, Davis; 'sk' indicates an isolate from S. T. Koike, UCCE, Salinas; isolate P-3295 provided by M. D. Coffey, University of California, Riverside; isolate R1-US6 by R. M. Bostock, University of California, Davis; Pr-104sz and P-43 by D. M. Rizzo, University of California, Davis; isolates MBA10, CacuSy and Mpha01 from authors of this work.

[b]–,no DNA fragment amplified; +, expected DNA fragment amplified; NT, not tested.

[c]Template DNA was subjected to a first round of PCR using ITS1 and ITS4 primers (White *et al.*, 1990), then diluted 1:100 before a second, nested round of PCR using the primer pair ADF1 and ADR1 (Lacourt *et al.*, 1997), which amplified a 520-bp DNA fragment.

[d]Template DNA was subjected to a first round of PCR using ITS1 and ITS4 primers, then diluted 1:100 before a second, nested round of PCR using the primer pair BPhycacL87FRG and BPhycacR87RRG, which amplified a robust 340-bp and a faint 480-bp DNA fragment, or the primer pair BPhycacL89FRG and BPhycacR176RRG, which amplified only a 431-bp fragment.

sterile clarified V8 juice broth (200 mL V8 juice, 2 g $CaCO_3$, 800 mL deionized water) and sterile potato dextrose broth (24 g infusions from potatoes and glucose, 1000 mL deionized water), respectively. The cultures were incubated for 4–6 days at 20–22°C before the DNA was extracted from the mycelia (*ca*. 150 mg) using the phenol-chloroform method of Lee & Taylor (1990).

Total DNA was extracted from *ca*. 150-mg samples of strawberry tissues by several described methods, including the following: phenol-chloroform (Lee & Taylor, 1990), hexadecyltrimethyl-ammonium bromide (CTAB) (Doyle & Doyle, 1987; Wangsomboondee & Ristaino, 2002) and NaOH lysis (Xin *et al*., 2003). In addition, several proprietary DNA extraction kits were used according to the manufacturers' instructions, including the ChargeSwitch gDNA Mini Tissue Kit (Invitrogen Life Technologies), the DNeasy Plant Mini Kit (QIAGEN Inc.), the FastDNA Kit (Bio101), the QIAamp DNA Stool Mini Kit (QIAGEN Inc.) and the Ultraclean Plant DNA isolation Kit (MO BIO Laboratories Inc.). In addition to its use alone, the CTAB extraction method was used in conjunction with pre- and post-DNA-extraction steps designed in this study to facilitate PCR with DNA from necrotic strawberry tissues. The supplementary steps included (i) incubating approximately 150 mg strawberry tissue in 1·5 mL 5% alconox (Alconox Inc.) for 12–18 h prior to DNA extraction and (ii) electrophoretic purification of the extracted DNA using a modified agarose-embedded technique (Moreira, 1998). For the latter, two 1-mL pipette tips (USA Scientific, Inc.) were stacked vertically and tightly, and the pointed end of the lower pipette tip was sealed with parafilm. Agarose (2% in 0·5× TAE buffer) was poured into the upper tip and filled the void between the stacked tips before solidifying. Then the upper pipette tip was removed and approximately 2 cm of the pointed end was cut off. Fifty microlitres of DNA solution were placed in the agarose-lined cavity of the lower, uncut tip and the cut tip was stacked in the original position. The joint of the two tips was sealed with parafilm, and the assembly was immersed in 0·5× TAE buffer and electrophoresed at 48 volts for 5 min in a gel box. The DNA suspension was then removed from the cavity for PCR analyses.

PCR conditions and primers

A nested PCR system targeting the ITS regions of the rRNA gene of *P. cactorum* was tested for function and specificity using genomic DNA extracted from mycelium of *Phytophthora* spp., mycelium of fungi (Table 1) and samples of diseased strawberry plants. The first-round PCR in 25 μL included 1× reaction buffer (10 mM Tris-HCl pH 8·0, 50 mM KCl, 1·5 mM $MgCl_2$, 0·1% Triton X-100), 1·5 mM $MgCl_2$, 0·2 mM dNTPs mix, 0·2 μM forward primer ITS1 (White *et al*. 1990), 0·2 μM reverse primer ITS4 (White *et al*., 1990), 2% polyvinylpyrrolidone (average molecular weight 40 000) (PVP40), 2·5 U *Taq* polymerase (Gene Choice), ultrapure distilled water (GIBCO, Invitrogen Corporation) to make up the reaction volume to 25 μL, and 1–50 ng undiluted stock DNA suspension. Amplifications were conducted in a GeneAmp PCR System 9700 (Applied Biosystems) thermal cycler using the following parameters: initial denaturing at 94°C for 5 min, then 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min with a final extension at 72°C for 10 min, followed by cooling to 4°C. Ten microlitres of the first-round PCR amplicons were separated by 1·4% agarose gel electrophoresis in 0·5× TAE buffer, and DNA bands were visualized by ethidium bromide (0·5 μg mL^{-1}) staining and UV illumination. A control reaction was used to verify PCR-conducive conditions for each genomic DNA sample used. The reaction conditions and sample materials used for the control were the same as those used to detect *P. cactorum*, except that for the control the bacterial-universal primers 63F (Marchesi *et al*., 1998) and 1401R (Nubel *et al*., 1996) were substituted for primers ITS1 and ITS4.

Products of the first-round of PCR were diluted with ultrapure distilled water 1:100 or 1:25 for mycelia or necrotic strawberry samples, respectively, before use as template in the second round of PCR. The second-round reaction mixture was the same as the first, except that the following were used: primers (0·2 μM each) intended to be specific for *P. cactorum* (instead of primers ITS1 and ITS4) (Table 2), 0·2% PVP40, 1·5 U *Taq* DNA polymerase, ultrapure distilled water to make up the reaction volume to 24 μL, and 1·0 μL diluted template from the first-round PCR. The second-round amplification

Table 2 Primers used in this study

Target organism(s)	Primer name	Direction	Primer sequence (5′–3′)	Size of amplicon/s	Reference
Oomycetes and fungi	ITS1	Forward	TCCGTAGGTGAACCTGCGG	830 to 870 bp for Oomycetes and 500 to 600 bp for fungi	White *et al*., 1990
	ITS4	Reverse	TCCTCCGCTTATTGATATGC		
Bacteria	63f	Forward	CAGGCCTAACACATGCAAGTC	1340 bp	Marchesi *et al*., 1998
	R-1401	Reverse	CGGTGTGTACAAGACCC		Nubel *et al*., 1996
Phytophthora cactorum	ADF1	Forward	TACTGTGGGGACGAAAGTCCT	520 bp	Lacourt *et al*., 1997
	ADR1	Reverse	CCGATTCAAAAGCCAAGCAACT		
Phytophthora cactorum	BPhycacL87FRG	Forward	CTTCGGCCTGAGCTAGTAGCT	340 bp and 480 bp	This study
	BPhycacR87RRG	Reverse	CAGTCGGTCCGAAAACCAG		
Phytophthora cactorum	BPhycacL89FRG	Forward	GGCTTCGGCCTGAGCTAGTAGCT	431 bp	This study
	BPhycacR176RRG	Reverse	CAACTTGCTACAATA		

conditions were the same as the first, except that an annealing temperature of 65°C was used. Ten microlitres of the second-round PCR products were separated by 1·4% gel electrophoresis in 0·5× TAE buffer, and DNA bands were visualized by ethidium bromide staining and UV illumination.

The second-round PCR primers were designed to be specific for *P. cactorum* based on analysis of DNA sequences of fragments amplified by ITS1 and ITS4 primers from genomic DNA of *P. cactorum*, *P. citricola* and *P. fragariae* var. *fragariae*, as well as analysis of the rRNA gene sequences available through the National Center for Biotechnology Information (NCBI) for other species of *Phytophthora*, *Pythium* species, *C. acutatum*, *V. dahliae* and *M. phaseolina*. From each of three isolates of *P. cactorum*, the first-round PCR products were cloned (TOPO TA 2·1 Cloning Kit, Invitrogen) and at least two clones from each isolate were sequenced in both directions (College of Biological Sciences, ^{UC}DNA Sequencing Facility, University of California, Davis). Similarly, the second-round nested PCR products were cloned and sequenced. All of the sequences were aligned using CLUSTALW, and the stretches of DNA sequences unique to *P. cactorum* at the 5' region of ITS1 and the 3' region of ITS2 were identified and used for designing specific primers. The candidate primers were purchased (Operon Biotechnologies Inc.) and tested in nested PCR, both as single primers and in pairs of several combinations.

Validation of PCR protocols

Three sets of strawberry plants were used for PCR validations. The first set included potted strawberry plants of cvs Diamante and Ventana inoculated with *P. cactorum* in a greenhouse using methods described previously (Bhat *et al*., 2006). One week after soil infestation and every week thereafter, the inoculated and control plants were subjected to soil flooding for 24 h. Between flooding periods, the plants were watered every 1–2 days, and the soil was allowed to drain freely. All plants were washed free of soil 8 weeks after inoculation, and the root systems were used for culture-based isolations and DNA extraction. The second set included plants collected from three commercial high-elevation nurseries in late October 2006. At each nursery, five strawberry plants of three different cultivars with symptoms of stunting, necrotic lesions, wilting or die-back were sampled; each cultivar was planted in a separate field area. The third set of test samples included diseased strawberry plants sent by fruit growers, nurserymen and farm advisors from commercial fruit-production fields for diagnosis of disease. The plants typically exhibited symptoms of stunting or plant decline associated with crown and/or root rot.

For the plants in all three sets, tissue pieces (typically necrotic and 1–2 mm in diameter) were cut from roots, crown and petioles of each plant. The tissue pieces within a given tissue source (i.e. from within a single plant and tissue type) were pooled, mixed and then subdivided randomly into two portions, one for immediate use in culture-based isolations and the other for storage at −20°C for subsequent DNA extraction. Five to 10 tissue pieces from each pooled sample were cultured on the following isolation media: PARP (Kannwischer & Mitchell, 1978) for isolating *Phytophthora* and *Pythium* spp.; CollPDA (Freeman & Katan, 1997) for isolating *C. acutatum*, NP-10 (Kabir *et al*., 2004) for isolating *V. dahliae*; and water agar with ampicillin (Mazzola, 1998) for isolation of several other fungi including *M. phaseolina*. The isolation plates were monitored for 1–3 weeks for incidence of the pathogens. For PCR analysis, approximately 150 mg of the sample portions preserved at −20°C were used for DNA extraction and purification as described above.

Results

DNA extraction and purification

The DNA suspensions extracted from mycelia of 16 species of *Phytophthora* and three species of fungi by the phenol-chloroform and CTAB methods had acceptable concentrations (50–150 μg mL^{-1} TE buffer) and absorbance ratios (A_{260}/A_{280} ratios of 1·75–2·0). In contrast, the suspensions extracted from necrotic strawberry tissues by the phenol chloroform, CTAB or NaOH methods, or by the proprietary DNA extraction kits, had poor absorbance ratios (A_{260}/A_{280} ratios of 0·8–1·10). In addition, when several samples extracted from the diseased plants were subjected to analysis at A_{220} to A_{600}, the results suggested that much of the A_{260} was from compounds other than DNA. Pre-DNA-extraction tissue incubation in alconox solution and electrophoretic purification in agarose were effective in combination with the CTAB protocol for DNA extraction, yielding acceptable DNA concentrations (15–250 μg mL^{-1} TE buffer) and absorbance ratios (A_{260}/A_{280} ratios of 1·4–1·6) of DNA.

Nested PCR for specific detection of *P. cactorum*

In the first round of PCR, using the standard specified reaction conditions, primers ITS1 and ITS4 amplified expected DNA fragments from genomic DNA extracted from mycelia of *Phytophthora* spp. and fungi (Fig. 1a). However, the same reaction conditions and primers did not amplify fragments detectably from total DNA extracted from healthy and diseased strawberry plants using the described methods (i.e. phenol-chloroform, CTAB and NaOH methods and proprietary kits) (data not shown). Furthermore, the control PCR reactions with universal bacterial primers failed to amplify products detectably from genomic DNA of healthy and diseased strawberry plants.

Incubation of the strawberry tissue samples in alconox solution before DNA extraction by the CTAB method, followed by further purification of the extracted DNA using the modified agarose-embedded technique, overcame the apparent PCR inhibition in DNA extracts from strawberry. Although DNA samples extracted and

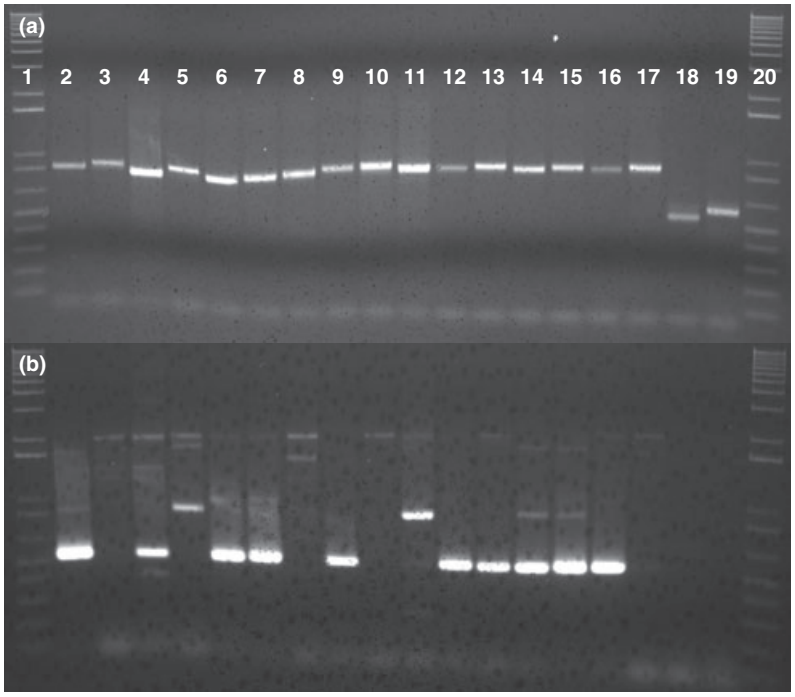

Figure 1 (a) DNA banding patterns after first-round amplification of ITS regions of rRNA gene of 16 *Phytophthora* species, *Verticillium dahliae* and *Colletotrichum acutatum* using primers ITS1 and ITS4. Fragment sizes from 530 to 870 bp were amplified. (b) DNA banding patterns after nested PCR for amplicons of the first-round PCR using primers designed for *P. cactorum* (Lacourt *et al.*, 1997), ADF1 and ADR1. Amplified DNA fragment from the first-round PCR was diluted 1:100, and 1 μL used as template DNA in the nested PCR. For both (a) and (b): lanes 1 and 20, 1-kb plus DNA ladder; lane 2, *P. cactorum*; lane 3, *P. cambivora*; lane 4, *P. capsici*; lane 5, *P. cinnamomi*; lane 6, *P. citricola*; lane 7, *P. citrophthora*; lane 8, *P. cryptogea*; lane 9, *P. drechsleri*; lane 10, *P. fragariae* var. *fragariae*; lane 11, *P. infestans*; lane 12, *P. lateralis*; lane 13, *P. megasperma*; lane 14, *P. nemorosa*; lane 15, *P. parasitica*; lane 16, *P. ramorum*; lane 17, *P. syringae*; lane 18, *V. dahliae*; and lane 19, *C. acutatum*.

purified in this manner from diseased strawberry plants did not always yield a visible product from the first round PCR with primers ITS1 an ITS4 (Fig. 2a), they consistently yielded a DNA fragment of 1340 bp following the control PCR with the bacterial-universal primers (Fig. 2b).

In the evaluations of primers for the second round of nested PCR using genomic DNA from mycelia, primers ADR1 and ADF1 did not amplify DNA visibly from *C. acutatum* or *V. dahliae*, but they amplified DNA fragments of 520 bp and other sizes non-specifically from *P. cactorum* and 15 other species of *Phytophthora* (Table 1, Fig. 1B). Similarly, most of the primers designed and tested in this study for the specific detection of *P. cactorum* were not specific for the pathogen when used either singly or in pairs in nested PCR (data not shown). However, the primers BPhycacL87FRG and BPhycacR87RRG (Table 2), specifically amplified a robust fragment of 340 bp and a second fragment of approximately 480 bp (Table 1, Fig. 3a). Cloning and sequencing confirmed that the 340- and 480-bp fragment sequences resided in the ITS1-5·8S-ITS2 region of *P. cactorum*. Primers BPhycacL87FRG and BPhycacR87RRG amplified faint bands of 340 and 480 bp from genomic DNA of *P. infestans*, but they did not amplify DNA of the other *Phytophthora* spp. or the fungi (Table 1). The second set of primers developed in this study, BPhycacL89FRG and BPhycacR176RRG (Table 2), amplified only one DNA band centered at 431 bp from *P. cactorum* (Fig. 3b), and cloning and sequencing confirmed that the band was amplified from the ITS1-5·8S-ITS2 region of *P. cactorum*. Primers BPhycacL89FRG and BPhycacR176RRG also

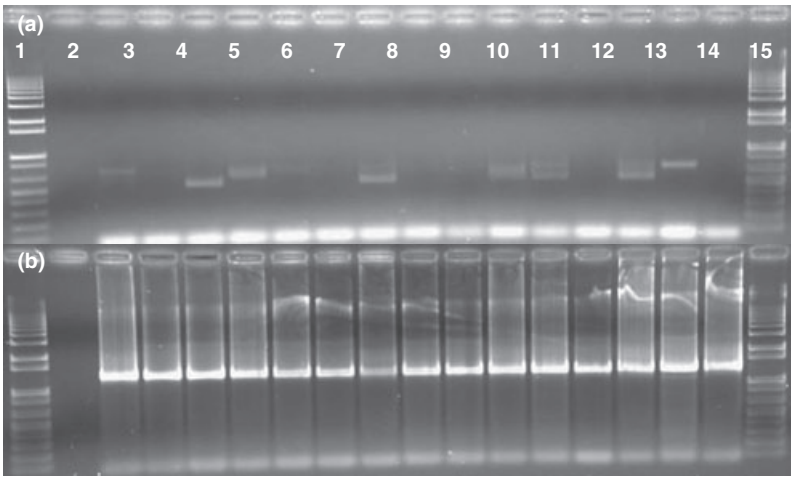

Figure 2 Representative DNA banding patterns after DNA extraction by the improved protocol (i.e. including tissue pre-soak in detergent and the agarose electrophoresis purification) and first-round amplification of (a) ITS regions of the rRNA gene using primers ITS1 and ITS4 and (b) bacterial conserved 16S region of the rRNA gene using primers 63F and 1401R. A 1340-bp DNA fragment was amplified by the latter primers as an indicator that the DNA preparations were free from PCR inhibitors. For both (a) and (b): lanes 1 and 15, 1-kb plus DNA ladder; lane 2, negative control (no DNA); lanes 3–14: total DNA from crown tissue pieces of diseased strawberry plants from commercial fruiting fields.

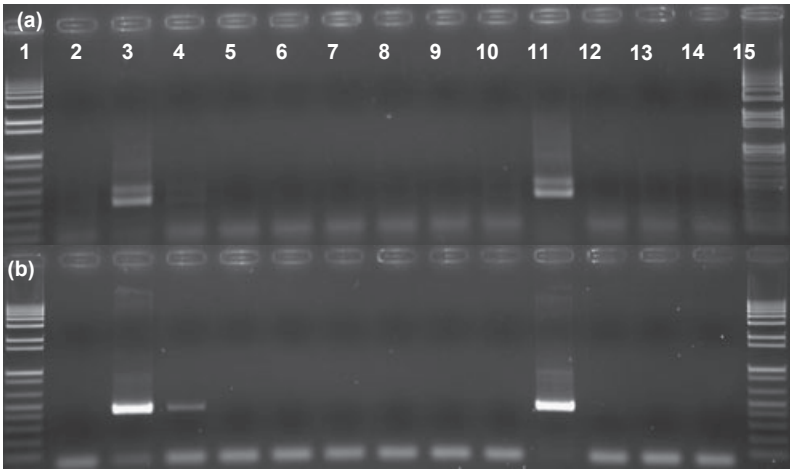

Figure 3 DNA banding patterns of nested PCR for specific detection of *Phytophthora cactorum* using two primer pairs. (a) Nested primers BPhycacL87FRG and BPhycacR87RRG amplified two fragments of 340 and 480 bp from amplicons of the ITS1-5·8S-ITS2 region of *P. cactorum*, and (b) nested primers BPhycacL89FRG and BPhycacR176RRG amplified a diagnostic fragment of 431 bp. For both (a) and (b): ITS regions of the rRNA gene were amplified in the first round PCR using primers ITS1 and ITS4. Lanes 1 and 15, 1-kb plus DNA ladder; lane 2, DNA from healthy strawberry crown; lanes 3–4, DNA from plants inoculated with *P. cactorum*; lanes 5–6, DNA from plants infected by *Colletotrichum acutatum*; lanes 7–8, DNA from plants infected by *Verticillium dahliae*; lanes 9–10, DNA from plants infected by *Macrophomina phaseolina*; lane 11, DNA from *P. cactorum*; lane 12, DNA from *P. citricola*; lane13, DNA from *V. dahliae*; lane 14, DNA from *C. acutatum.*

amplified a faint 431-bp band from *P. infestans*, but they did not amplify DNA from the other *Phytophthora* spp. or the fungi (Table 1). In the evaluations of primer pairs using total DNA samples extracted from diseased strawberry plants, the primers ADR1 and ADF1 amplified a 520-bp DNA fragment from samples that culture-based isolations indicated were not infected with *P. cactorum* (data not shown). In contrast, the primer pairs BPhycacL87FRG/BPhycacR87RRG and BPhycacL89FRG/BPhycacR176RRG amplified in agreement with results of culture-based isolations; i.e. the DNA fragment sizes described for these primer sets were amplified from strawberry root, crown and petiole samples confirmed by culturing to be infected with *P. cactorum*, and no DNA fragments were amplified visibly from samples that failed to yield *P. cactorum* in culture. Furthermore, the agreement between culture-based results and PCR-based results held when non-target fungi (i.e. *C. acutatum*, *V. dahliae*, *M. phaseolina*, species of *Alternaria*, *Aspergillus, Botrytis, Cylindrocarpon*, *Fusarium*, *Mortierella*, *Penicillium, Pythium*, *Trichoderma* and others) were detected in the samples by culturing; i.e. the expected nested PCR products with primer sets BPhycacL87FRG/BPhycacR87RRG or BPhycacL89FRG/BPhycacR176RRG were produced from strawberry tissue samples when *P. cactorum* was cultured, but the products were not produced when *P. cactorum* was not isolated, regardless of the culture-indicated presence of the non-target pathogenic and saprophytic fungi.

Diagnostic validation of PCR protocols

In validation assays using the improved DNA extraction and purification protocols and nested PCR with primers BPhycacL87FRG and BPhycacR87RRG, the results of PCR and culture-based isolations were in general agreement. In the first assay, which involved plants of strawberry cvs Diamante and Ventana inoculated with *P. cactorum* and non-inoculated controls in a greenhouse, the PCR-based detections of *P. cactorum* matched with culture-based detections of the pathogen (Table 3). Using both detection methods, the pathogen was detected in all inoculated plants and not in controls. In the second validation assay, which involved 45 plants from three commercial nurseries, both PCR and culture isolations detected *P. cactorum* only in plants of cv. Diamante from nursery 2 (Table 4). Plants of other cultivars (Camino Real and Sabrosa) from the same nursery did not yield *P. cactorum*. Similarly, *P. cactorum* was absent in all plants of the five cultivars from nurseries 1 and 3, except in two plants from nursery 3, in which PCR detected *P. cactorum* in DNA from root and petiole tissue in cvs Albion and Camarosa, respectively (Table 4). Culture-based isolations did not detect *P. cactorum* in the latter two plants. In the third validation assay, *P. cactorum* was detected in 26 of 98 diseased plants by culturing and 25 of 98 diseased plants by PCR (Table 4). Occasionally, both conventional plating assays and nested PCR detected *P. cactorum* in only certain parts of the diseased strawberry plants.

Valiadations also were completed as described above for the second set of nested primers, BPhycacL89FRG and BPhycacR176RRG, except that only a subset of 90 samples from those described above were used. The subset included four negative and six positive samples from the greenhouse inoculation study as well as 74 negative and six positive samples from the nursery and fruit-production field assay. For these samples the results with

Table 3 Comparative detection of *Phytophthora cactorum* using culture-based assays and nested PCR with samples of strawberry plants from a greenhouse experiment

			Number of plants determined to be infected by *P. cactorum*					
			Root		Crown		Petiole	
Treatment[a]	Cultivar	No. of plants	Plating[b]	PCR[c]	Plating	PCR	Plating	PCR
Control	Diamante	3	0	0	0	0	0	0
	Ventana	3	0	0	0	0	0	0
P. cactorum	Diamante	5	5	5	5	5	5	5
	Ventana	3	3	3	3	3	3	3

[a]University of California (UC) mix soil infested with or without *P. cactorum*.
[b]Isolation medium used for plating assays was PARP for *Phytophthora cactorum*.
[c]DNA from strawberry tissue was subjected to first-round PCR using ITS1 and ITS4 primers, then diluted 1:25 before a second round of PCR with the *P. cactorum*-specific nested primers, BPhycacL87FRG and BPhycacR87RRG, to amplify DNA fragments of 340 and 480 bp.

Table 4 Comparative detection of *Phytophthora cactorum* using culture-based assays and nested PCR with samples of declining strawberry plants from commercial sources

		Number of *P. cactorum*-positive plants					
		Root		Crown		Petiole	
Cultivar	No. of plants	Plating[a]	PCR[b]	Plating	PCR	Plating	PCR
Commercial nurseries							
Camino Real (nursery 1)	5	0	0	0	0	0	0
Diamante (nursery 1)	5	0	0	0	0	0	0
Ventana (nursery 1)	5	0	0	0	0	0	0
Camino Real (nursery 2)	5	0	0	0	0	0	0
Diamante (nursery 2)	5	3	4	4	5	2	2
Sabrosa (nursery 2)	5	0	0	0	0	0	0
Albion (nursery 3)	5	0	1	0	0	0	0
Camarosa (nursery 3)	5	0	0	0	0	0	1
Ventana (nursery 3)	5	0	0	0	0	0	0
Fruit-production fields							
Albion	18	0	0	0	0	0	0
Aromas	1	0	0	1	1	0	0
Camarosa	21	NT[d]	NT	0	0	NT	NT
CG-5	1	0	0	0	0	0	0
CG-6	8	0	0	0	0	0	0
Diamante	20	10	9	16	15	NT	NT
Unknown	4	2	2	4	4	2	2
Ventana	25	NT	NT	5	5	5	5

[a]Isolation medium for plating assays was PARP for *Phytophthora cactorum*.
[b]DNA from strawberry tissue was subjected to first-round PCR using ITS1 and ITS4 primers, then diluted 1:25 before a second round of PCR with the *P. cactorum*-specific nested primers, BPhycacL87FRG and BPhycacR87RRG, to amplify DNA fragments of 340 and 480 bp.

BPhycacL89FRG and BPhycacR176RRG were identical to those with BPhycacL87FRG and BPhycacR87RRG, except that the former primer pair amplified one 431-bp band from *P. cactorum* instead of the 340- and 480-bp bands.

Discussion

Novel DNA purification steps and pathogen-specific primers for detection of *P. cactorum* in root, crown and petiole tissues of strawberry plants from commercial nurseries and fruiting fields were developed and validated. The DNA purification steps should prove valuable for investigators and diagnosticians attempting to overcome PCR inhibition in necrotic strawberry tissues. The primer sets developed in this study, BPhycacL87FRG and BPhycacR87RRG, and BPhycacL89FRG and BPhycacR176RRG, were each validated using controlled, inoculated samples, as well as a large number of diseased commercial strawberry plants, and therefore they may be valuable additions to the few primer sets currently available for *P. cactorum* (Lacourt *et al.*, 1997; Causin *et al.*, 2005; Schena *et al.*, 2008).

The specific compounds responsible for the PCR inhibition that the novel DNA purification steps overcame are unknown, but it has been shown that plant polyphenols and polysaccharides, which are present in abundance in necrotic plant tissues, can act as PCR inhibitors

(Wilson, 1997; Watson & Blackwell, 2000). Difficulties in obtaining inhibitor-free DNA from *Phytophthora*-infected plants by using different DNA extraction procedures and kits have been discussed previously (Grote *et al*., 2002).

Although the first nested primer pair, BPhycacL87FRG and BPhycacR87RRG, was designed to amplify only one fragment of 340 bp based on the sequence of the ITS1-5·8S-ITS2 region of *P. cactorum*, it always produced two fragments of 340 and 480 bp. When several fragments of each size were cloned and sequenced, the sequences suggested that the longer fragment had resulted from one of the following: priming at a non-intended target of similar sequence, sequence variation among copies of the rRNA gene of *P. cactorum*, or primer concatenations or fragment chimeras. When several fragments were cloned and sequenced from the 431-bp band produced by primers BPhycacL89FRG and BPhycacR176RRG, all of the fragments shared homology with the intended target region, but sequence variation in the priming regions suggested that imperfect priming had occurred for some fragments. The imperfect priming may have resulted from sequence variations among rRNA gene copies and/or from PCR thermodynamics. An essential point concerning the amplification of double bands and variation in priming sites is that neither prevented the primer sets developed in this study from being specific for *P. cactorum* in strawberry. The fact that both of the primer pairs developed in this study amplified faint bands from DNA of *P. infestans* is not considered to be a major problem because the pathogen is not known to affect strawberry and would not be expected to occur on strawberry except as an unlikely surface contaminant.

Nested PCR was used to detect *P. cactorum* in diseased strawberry plants because of its sensitivity and flexibility. It was reported that nested PCR increased the sensitivity of detection for *P. melonis* 1000-fold, compared to a single-round, non-nested PCR (Wang *et al*., 2007). Nesting also facilitates use of relatively non-specific PCR primers in the first round of PCR for amplification of multiple pathogens, followed by use of pathogen-specific primers in the second round of PCR. This two-step PCR strategy has been used for the detection of *P. fragariae* var. *fragariae*, *C. acutatum*, *V. dahliae* and *M. phaseolina* affecting strawberry (R. G. Bhat & G. T. Browne, unpublished data). A disadvantage of nested PCR is that it is not useful for quantifying target DNA concentrations. Also, nested PCR can be sensitive to DNA contaminants, so appropriate precautions (Coleman & Tsongalis, 1997) should be taken. Although culture-based isolations and a PCR-based system detected *P. cactorum* with nearly identical sensitivity in the validation assays in this study, the PCR method offers many advantages over culturing for routine diagnosis. Compared to culturing, the PCR methods developed here are much more rapid and easy to use for individuals without specialized mycological training. The time required for the PCR assay of 12 samples by a single operator is estimated to be approximately 12 h distributed over 2 days, compared with a similar amount of time distributed over 10 days for culturing. Finally, DNA extracted from diseased strawberry plants is useful for PCR assays for multiple pathogens, which offers additional but harder-to-measure savings in time.

Results indicate that the DNA-purification and PCR-detection systems developed here are robust and worthy of secondary evaluations in commercial and research applications. The three sets of strawberry samples used in diagnostic validations harboured various fungi, bacteria and other microflora in necrotic tissues of several different strawberry cultivars in various states of decline, yet DNA was consistently and specifically amplified from the target pathogen, *P. cactorum*. Although not attempted in this study, the methods, primers and reaction conditions reported here may be adaptable for real-time PCR.

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References

Amouzou-Alladaye E, Dunez J, Clerjeau M, 1988. Immunoenzymatic detection of *Phytophthora fragariae* in infected strawberry plants. *Phytopathology* **78**, 1022–6.

Atkins SD, Clark IM, 2004. Fungal molecular diagnostics: a mini review. *Journal of Applied Genetics* **45**, 3–15.

Bhat RG, Browne GT, 2007. Development of an improved PCR-based technique for detection of *Phytophthora cactorum* in strawberry plants. *Phytopathology* **97**, S10, Abstract.

Bhat RG, Colowit PM, Tai TM, Aradhya MK, Browne GT, 2006. Genetic and pathogenic variation in *Phytophthora cactorum* affecting fruit and nut crops in California. *Plant Disease* **90**, 161–9.

Bonants P, Hagenaar-de Weerdt M, Vam Gent-Pelzer M, Lacourt I, Cooke D, Duncan J, 1997. Detection and identification of *Phytophthora fragariae* Hickman by the polymerase chain reaction. *European Journal of Plant Pathology* **103**, 345–55.

Bonants PJM, Van Gent-Pelzer MPE, Hooftman R, Cooke DEL, Guy DC, Duncan JM, 2004. A combination of baiting and different PCR formats, including measurement of real-time quantitative fluorescence, for the detection of *Phytophthora fragariae* in strawberry plants. *European Journal of Plant Pathology* **110**, 689–702.

Causin R, Scopel C, Grendene A, Montecchio L, 2005. An improved method for the detection of *Phytophthora cactorum* (LC) Schroeter in infected plant tissues using scar markers. *Journal of Plant Pathology* **87**, 25–35.

Coleman WD, Tsongalis GJ. 1997. The polymerase chain reaction. In: Coleman WB, Tsongalis GJ, eds. *Molecular Diagnostics for the Clinical Laboratorian*. Totowa, NJ, USA: Humana Press, Inc., 47–55.

Doyle JJ, Doyle JL, 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* **19**, 11–5.

Freeman S, Katan T, 1997. Identification of *Colletotrichum* species responsible for anthracnose and root necrosis of strawberry in Israel. *Phytopathology* **87**, 516–21.

Grote D, Olmos A, Kofoet A, Tuset JJ, Bertolini E, Cambra M, 2002. Specific and sensitive detection of *Phytophthora nicotianae* by simple and nested-PCR. *European Journal of Plant Pathology* **108**, 197–207.

Hughes KJD, Inman AJ, Beales PA, Cook RTA, Fulton CE, McReynolds ADK, 1998. PCR-based detection of *Phytophthora fragariae* in raspberry and strawberry roots. In: *Brighton Crop Protection Conference: Pests and Diseases, November 16–19, 1998, Brighton, UK*, vol. 2. 687–92.

Ioos R, Laugustin L, Schenck N, Rose S, Husson C, Frey P, 2006. Usefulness of single copy genes containing introns in *Phytophthora* for the development of detection tools for the regulated species *P. ramorum* and *P. fragariae*. *European Journal of Plant Pathology* **116**, 171–6.

Kabir Z, Bhat RG, Subbarao KV, 2004. Comparison of media for recovery of *Verticillium dahliae* from soil. *Plant Disease* **88**, 49–55.

Kannwischer ME, Mitchell DJ, 1978. Influence of a fungicide on the epidemiology of black shank of tobacco. *Phytopathology* **68**, 1760–5.

Lacourt I, Bonants PJM, Van Gent-Pelzer MP *et al.*, 1997. The use of nested primers in the polymerase chain reaction for the detection of *Phytophthora fragariae* and *P. cactorum* in strawberry. *Acta Horticulturae* **439**, 829–38.

Lane CR, Hobden E, Walker L *et al.*, 2007. Evaluation of a rapid diagnostic field test kit for identification of *Phytophthora* species, including *P. ramorum* and *P. kernoviae* at the point of inspection. *Plant Pathology* **56**, 828–35.

Lee SB, Taylor JW, 1990. Isolation of DNA from fungal mycelia and single spores. In: Innes MA, Gelfand DH, Sninsky JJ, White TJ, eds. *PCR Protocols: A Guide to Methods and Applications*. San Diego, CA, USA: Academic Press, 282–7.

Maas JL, 1998. *Compendium of Strawberry Diseases*. St Paul, MN, USA: APS Press.

MacDonald JD, Stites J, Kabashima J, 1990. Comparison of serological and culture plate methods for detecting species of *Phytophthora*, *Pythium*, and *Rhizoctonia* in ornamental plants. *Plant Disease* **74**, 655–9.

Marchesi JR, Sato T, Weightman AJ et al., 1998. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Applied and Environmental Microbiology* **64**, 795–9.

Mazzola M, 1998. Elucidation of the microbial complex having a causal role in the development of apple replant disease in Washington. *Phytopathology* **88**, 930–8.

Moreira D, 1998. Efficient removal of PCR inhibitors using agarose-embedded DNA preparations. *Nucleic Acids Research* **26**, 3309–10.

Nubel U, Engelen B, Felske A *et al.*, 1996. Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. *Journal of Bacteriology* **178**, 5636–43.

Olsson CHB, 1995. Diagnosis of *Phytophthora* infections in raspberry and strawberry plants by ELISA tests. *Journal of Phytopathology* **143**, 307–10.

Schena L, Duncan JM, Cooke DEL, 2008. Development and application of a PCR-based 'molecular tool box' for the identification of *Phytophthora* species damaging forests and natural ecosystems. *Plant Pathology* **57**, 64–75.

Strand L, 1994. *Integrated Pest Management for Strawberries*. California, USA: University of California, Publication 3351.

Wang Y, Ren Z, Zheng XB, Wang YC, 2007. Detection of *Phytophthora melonis* in samples of soil, water, and plant tissue with polymerase chain reaction. *Canadian Journal of Plant Pathology* **29**, 172–81.

Wangsomboondee T, Ristaino JB, 2002. Optimization of sample size and DNA extraction methods to improve PCR detection of different propagules of *Phytophthora infestans*. *Plant Disease* **86**, 247–53.

Watson RJ, Blackwell B, 2000. Purification and characterization of a common soil component which inhibits the polymerase chain reaction. *Canadian Journal of Microbiology* **46**, 633–42.

White TJ, Bruns T, Lee S, Taylor J, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innes MA, Gelfand DH, Sninsky JJ, White TJ, eds. *PCR Protocols: A Guide to Methods and Applications*. San Diego, CA, USA: Academic Press, 315–22.

Wilson IG, 1997. Inhibition and facilitation of nucleic acid amplification. *Applied and Environmental Microbiology* **63**, 3741–51.

Xin Z, Velten JP, Oliver MJ, Burke JJ, 2003. High-throughput DNA extraction method suitable for PCR. *BioTechniques* **34**, 820–5.