Enhanced Polymerase Chain Reaction Methods for Detecting and Quantifying *Phytophthora infestans* in Plants

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ABSTRACT

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Sensitive and specific primer sets for polymerase chain reaction (PCR) for *Phytophthora infestans*, the oomycete that causes late blight of potato and tomato, were developed based on families of highly repeated DNA. The performance of these primers was compared to those developed previously for the internal transcribed spacer (ITS) of ribosomal DNA. The detection limit using the new primers is 10 fg of *P. infestans* DNA, or 0.02 nuclei. This is about 100 times more sensitive than ITS-directed primers. Nested polymerase chain reaction (PCR) allows the measurement of down to 0.1 fg of DNA using the new primers. To enhance the reliability of diagnostic assays, an internal positive control was developed using an amplification mimic. The mimic also served as a competitor for quantitative PCR, which was used to assess the growth of *P. infestans* in resistant and susceptible tomato. A key dimension of this study was that two laboratories independently checked the specificity and sensitivity of each primer set; differences were noted that should be considered when PCR is adopted for diagnostic applications in any system.

Late blight of potato and tomato has become increasingly severe due to the appearance of fungicide-resistant and aggressive genotypes of the pathogen, *Phytophthora infestans* (Mont.) de Bary (11). Many strategies for controlling late blight involve detecting or quantifying *P. infestans* within plant tissue. Checking seed potatoes and tomato transplants for infection is a priority since these are sources of inoculum (1,11). Many seed certification agencies in the United States accordingly established maximum infection tolerances of 0 to 1% for tubers. Postharvest detection of late blight is also important, as a few infected tubers can compromise an entire storage facility (26). Studies of the disease cycle and resistance breeding programs also benefit from accurate measurements of pathogen growth (7).

Traditional methods for detecting *P. infestans* involve visual examination or culturing from plant tissue. However, visual inspection misses outwardly asymptomatic infections and is confounded by the similarity of symptoms caused by *P. infestans* and other pathogens. Isolating *P. infestans* is difficult when secondary microflora or other species of *Phytophthora* are present, and involves a timeframe incompatible with realities of the marketplace. Immunodetection is potentially rapid but existing antibodies for *P. infestans* lack specificity (12). Strains of *P. infestans* with marker transgenes may aid experimental studies of pathogen growth (16) and host resistance (17), but regulations for transgenics limit such applications.

Polymerase chain reaction (PCR) is now established as an important technique for detecting pathogens, offering higher sensitivity and specificity than many traditional methods (13). PCR protocols for detecting various *Phytophthora* spp. targeted intergenic spacer regions (ITS) of ribosomal DNA (3,19,24,25,29,32) and other low-to-middle copy sequences (4,8,18,22). Such assays reported detection thresholds near the picogram range and varying levels of species specificity.

The impetus for the present study was the identification of DNA sequences from *P. infestans* that existed at exceptionally high copy numbers and were species-specific based on hybridiza-

TABLE 1. Isolates of *Phytophthora* tested in polymerase chain reaction assays

Name[a]	Mating type	Origin	Place and year isolated
115.11	A2	*Solanum tuberosum*	Canada; 1994
127 (US-1)	A1	*Solanum tuberosum*	United States; 1982
198 (US-1)	A1	*Solanum tuberosum*	United States; 1994
199 (US-8)	A2	*Solanum tuberosum*	United States; 1994
510	A2	*Solanum tuberosum*	Mexico; 1983
511	A1	*Solanum tuberosum*	Mexico; 1983
529	A1	*Solanum tuberosum*	Mexico; 1983
541	A1	*Solanum tuberosum*	Mexico; 1983
550	A2	*Solanum stoloninferum*	Mexico; 1983
580	A1	*Solanum tuberosum*	Mexico; 1983
582	A1	*Solanum tuberosum*	Mexico; 1983
618	A2	*Solanum tuberosum*	Mexico; 1987
654	A2	*Solanum tuberosum*	Mexico; 1987
1114	A1	*Solanum tuberosum*	Netherlands; 1985
1163	A1	*Solanum tuberosum*	Poland; 1985
1296	A1	*Solanum tuberosum*	United Kingdom; –[b]
1306	A1	*Lycopersicon esculentum*	United States; 1982
1362	A1	*Solanum tuberosum*	Mexico; 1979
1363	A1	*Solanum tuberosum*	Mexico; 1979
1389	A1	*Lycopersicon esculentum*	United States; 1980
1484	A1	*Solanum tuberosum*	United Kingdom; 1982
6150	A1	*Solanum tuberosum*	Japan; 1988
6162	A1	*Solanum tuberosum*	Japan; 1988
6170	A2	*Solanum tuberosum*	Japan; 1988
6736	A1	*Solanum tuberosum*	Rwanda; 1985
7629 (US-6)	A1	*Solanum tuberosum*	United States; –
7722	A1	*Lycopersicon esculentum*	United States; 1992
7723 (US-7)	A2	*Lycopersicon esculentum*	United States; 1992
8811	A1	*Solanum tuberosum*	United Kingdom; 1988
93H3 (US-7)	A2	*Lycopersicon esculentum*	United States; 1993
E13a	A2	*Solanum tuberosum*	Egypt; 1984

[a] The U.S. pathogen lineage according to the nomenclature of Fry and Goodwin (11) is indicated where known.
[b] Unrecorded.

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tion assays (15). This paper describes the use of such sequences to develop primers for PCR that proved to be about 100 times more sensitive than those previously applied to *P. infestans*. Enhancements not generally described for other *Phytophthora*-directed PCR assays are also introduced, such as an internal control useful for diagnostic tests and a competitor-based method for in planta quantification. An additional dimension of this study was a comparison of the various primer sets in different laboratories.

MATERIALS AND METHODS

Sources of DNA. DNA was extracted from pure microbial cultures as described (16,29). These cultures included isolates of *P. infestans*, other species of *Phytophthora*, and other genera as listed in Tables 1, 2, and 3, respectively. DNA from uninfected tomato or potato leaflets was obtained by CTAB extraction (21). DNA from tomato leaflets or potato tubers infected with zoospores of *P. infestans* (16,29) was isolated by sodium hydroxide and Qiagen (Valencia, CA) ion-exchange methods, respectively (29). DNA concentrations were determined with spectrophotometric or dye-binding assays.

Oligonucleotides. PCR primers are listed in Table 4. PINF2, ITS3, PINF, and ITS5 were as described (29,32). Primers based on DNA families AE7 and O8 (15) were designed using Oligo 4.03 (National Biosciences, Plymouth, MN). Primers were purchased by the Maryland (MD) Laboratory from Life Technologies (Gaithersburg, MD), and by the California (CA) Laboratory from Genosys (The Woodlands, TX) or Operon Technologies (Alameda, CA).

Amplifications. Reactions in CA and MD were performed in thermal cyclers from MJ Research (Model PTC-100; Waltham, MA) and Perkin-Elmer (Model 9600; Norwalk, CT), respectively, in 25 µl using thin-walled vessels. In CA, reactions were incubated 30 s at 94°C, cycled 35 times between 94°C (30 s), annealing temperatures of 50°C (30 s for O8 and AE7 primers) or 55°C (30 s, for PINF2-ITS3 or PINF-ITS5 primer sets), and 72°C (60 s), and then held for 4 min at 72°C before cooling to room temperature. The same parameters were used in MD but each step lasted 15 s, except for the terminal extension which was maintained at 4 min. These times were selected based on the experience of each laboratory, since the rates of heating and cooling varies depending on the instrument and reaction vessels being used.

PCR of O8 and AE7 sequences was performed in 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 0.01% gelatin, 1.8 mM $MgCl_2$, 0.1 mM deoxynucleotide triphosphates (dNTPs), 0.4 µM each primer, and 1 unit of *Taq*DNA polymerase. The latter was purchased by the

TABLE 2. Isolates of *Phytophthora* other than *P. infestans*, tested in polymerase chain reaction assays

Species	Origin	Place and year isolated[a]	Name[b]
P. boehmeriae	*Capiscum annum* (pepper)	Pakistan; 1989	7472
P. cactorum	*Malus pumila* (apple)	Germany;1975	3406
	Malus pumila (apple)	United States (NY); –	385
P. capsici	*Leucospermum* spp.	United States (HA); 1989	6520
	Capsicum spp. (pepper)	United States (FL); 1983	302
P. cinnamomi	*Anasus comosus* (pineapple)	Taiwan; 1989	6379
P. citricola	*Rubus idaeus* (raspberry)	United States (CA); –	1321
P. citrophthora	*Theobroma cacao* (cocoa)	Brazil; –	1200
P. colocasiae	*Theobroma cacao* (cocoa)	Cameroon; 1964	6012
	Colocasiae esculenta (taro)	China; –	345
P. cryptogea	*Prunus avium* (cherry)	United States (CA); –	389
P. dreschleri	*Cajanus cajan* (pigeon pea)	India; –	1795
	Solanum tuberosum (potato)	Egypt; 1985	401
P. erythroseptica	*Solanum tuberosum* (potato)	Ireland; 1989	6180
	Solanum tuberosum (potato)	United States (ME); 1996	374
P. fragariae	*Fragaria* spp. (strawberry)	United States (OR); 1989	3569
	Fragaria spp. (strawberry)	United States (MD); –	394
P. heaveae	*Theobroma cacao* (cocoa)	Malaysia; 1980	3604
P. hibernalis	*Citrus cinensis* (sweet orange)	United States (CA); 1991	7297
	Citrus cinensis (sweet orange)	United States (CA); –	337
P. humicola	*Phaseolis vulgaris* (bean)	Taiwan; –	6701
P. ilicis	*Ilex aquifolium* (holly)	Canada; –	3939
	Ilex aquifolium (holly)	United States (OR); –	343
P. inflata	*Syringa vulgaris* (lilac)	England; 1990	7492
P. insolita	Soil	Taiwan; 1979	6195
P. iranica	*Solanum melongena* (eggplant)	Iran; 1969	3882
P. katsurae	*Cocos nucifera* (coconut)	United States (HA); 1982	6921
P. lateralis	*Chamaecyparis* spp. (cedar)	United States (OR); 1988	3888
P. meadii	*Hevea brasiliensis* (rubber)	India; 1958	6503
P. megasperma	*Asparagus officinalis* (asparagus)	France; 1989	6616
	Pseudotsuga menziesii (Douglas fir)	United States (OR); –	309
P. mirabilis	*Mirabilis jalapa* (four o'clock)	Mexico; 1985	3010
	Mirabilis jalapa (four o'clock)	Mexico; 1985	340
P. nicotiana	*Nicotiana tabacum* (tobacco)	United States (NC); –	331
P. palmivora	*Carica papaya* (papaya)	United States (HA); –	1787
P. parasitica	*Citrus* spp. (orange)	United States (CA); –	1582
P. phaseoli	*Phaseolus lunatus* (lima bean)	United States (DE); 1989	352
	Phaseolus lunatus (lima bean)	United States (MD); 1996	373
P. porri	*Allium sepa* (onion)	United States (CA); 1993	7979
P. quininea	*Cinchona officinalis* (quinine)	Peru; 1947	8488
P. richardiae	*Zantedeschia aethiopica* (lily)	United States (UN); 1930	3876
P. sojae	*Glycine max* (soybean)	United States (MS); –	7076
P. tentaculata	*Chrysanthemum leucanthemum* (daisy)	Germany;1975	8497
P. vignae	*Vigna sinensis* (cowpea)	Australia; approximately 1960	3018

[a] United States locations include two-letter state designator or unknown (UN); – indicates unrecorded.

[b] Isolates with three-digit designations are from the authors' collections. Isolates with four-digit names are from the collection of M. Coffey, University of California, Riverside.

CA and MD laboratories from Promega (Madison, WI) and PE Biosystems (Forster City, CA), respectively. Reaction conditions using PINF2 with ITS3, and PINF with ITS5, were as recommended by Tooley et al. (29) and Trout et al. (32). These differed from those listed above as PINF2 and ITS3 were used at 0.1 μM with 0.2 mM dNTPs, and PINF plus ITS5 were used with 0.08 mM dNTPs and 1.9 mM $MgCl_2$.

Amplification mimics were obtained by subjecting DNA from *E. coli* strain DH5α to PCR with primers O8-3 and O8-4 using an annealing temperature of 35°C. Reaction products were cloned into pGEMT Easy (Promega).

Gel electrophoresis and imaging. PCR products were resolved by electrophoresis in gels generally containing 1.4 to 2% agarose, or 0.75% Infinity Agarose Enhancer (Oncor, Gaithersburg, MD) –0.5% agarose, in Tris-Borate-EDTA buffer (90 mM Tris-Borate and 2 mM EDTA) and stained with ethidium bromide. Images were captured digitally or on film. Quantification was performed using images recorded by a Fluor-S CCD system with Quantity One software (Bio-Rad, Hercules, CA).

RESULTS

Selection of amplification targets. A study of repeated DNA in *P. infestans* suggested that families O8 and AE7 represented useful targets for sensitive and specific PCR (15). This was because these families contained 14,000 and 12,000 members per nucleus, respectively. Also, clones representative of each family did not cross-hybridize to DNA from other members of the genus besides *P. mirabilis* and *P. phaseoli*. These species, plus *P. infestans*, are known to be closely related based on morphological (33) and DNA markers (29). The sequence of the O8 element displays weak similarity to reverse transcriptases (BLAST $E = 6 \times 10^{-2}$), suggesting its evolution from a retroelement, and appeared to be dispersed throughout the genome (15). In contrast, AE7 showed no informative matches in database searches and exists in the genome as a moderately degenerate tandem repeat with a monomer size of 5.6 kb.

Amplifications using two primer sets for O8 and one for AE7 (Table 4) revealed that the targets were well-conserved within *P. infestans*. The expected amplicons were obtained in each of 31 genetically and geographically diverse isolates tested (Fig. 1). Larger bands also frequently amplified, suggesting that some individual copies of the sequences existed in different conformations within each nucleus.

Sensitivity of PCR. Of the O8, AE7, and ITS-based primers (30,32), the former were most sensitive at detecting DNA of *P. infestans* based on data from both the CA and MD laboratories (Fig. 2). Primers targeted against O8 were ~100× more sensitive than ITS primers, displaying a detection limit near 10 fg of genomic DNA. This is equivalent to 0.02 nuclei (31). In these amplifications and subsequent tests of specificity, the reaction conditions (including reagent composition and annealing temperatures) used for the ITS primers were as previously recommended (30,32) and differed slightly from the conditions used for the O8 and AE7 primers as described previously.

A further increase in sensitivity was enabled by nested PCR against O8, which was possible since primer O8-3 bound within the O8-1/O8-4 amplicon. In four replicate nested assays, 1 fg of

TABLE 3. Saprophytes and plant pathogens tested in polymerase chain reaction assays

Species	Origin	Place and date isolated
Oomycetes		
Achlya bisexualis	Pond water	United States (NY); –[a]
Halophytophthora spinosa 3823	*Rhizophora*	Vietnam; 1972
Lagenidium giganteum	Mosquito	–; –
Pythium ultimum PY-4	Potato	United States (ME); 1994
Pythium ultimum PY-5	Potato	United States (ME); 1994
Saprolegnia monoica	Pond water	–; –
True fungi		
Alternaria solani AS-1	Potato	United States (FL); 1993
Fusarium oxysporum FS-1	Potato	United States (FL); 1994
Fusarium sambucinum FS-3	Potato	United States (ID); 1992
Helminthosporium solani HS-2	Potato	Canada; 1995
Helminthosporium solani HS-4	Potato	Canada; 1995
Rhizoctonia solani RZ-1	Potato	United States (ME); 1995
Trichoderma harzianum K4	Soil	United States (CA); –
Verticillium albo-atrum VAA-1	Potato	United States (MN); 1994
Verticillium dahliae VD-1	Potato	United States (MN); 1995
Bacteria		
Clavibacter michiganensis spp. *sepedonicus*	Potato	United States (ID); 1998
Erwinia carotovora ssp. *Carotovora* ECC-1	Potato	United States (CO); 1990
Erwinia chrysanthemi ECH-1	Potato	United States (CO); 1990
Escherichia coli DH5α	–	–; –
Pseudomonas solanacerum PSOL-1	Potato	United States (FL); 1993
Pseudomonas syringae pv. *tomato* T23	Tomato	United States (CA); 1985

[a] Unrecorded, unknown, or not relevant.

TABLE 4. Primers used in this study

Primer set	Primer sequences (5′ to 3′)		Major amplicon from *Phytophthora infestans*
O8-1, 08-2	AAGATGATGTTGGATGATTG	TGCCTGATTTCTACCTTCT	245 bp
O8-3, 08-4	GAAAGGCATAGAAGGTAGA	TAACCGACCAAGTAGTAAA	258 bp
AE7-1, AE7-2	GCCGCCGACATATTGAAT	CAAATCTGCGAACGAGACAT	171 bp
PINF2, ITS3[a]	CGATTCAAATGCCAAGCTAAAG	GCATCGATGAAGAACGCAGC	456 bp
PINF, ITS5[b]	CTCGCTACAATAGGAGGGTC	GGAAGTAAAAGTCGTAACAAGG	600 bp

[a] Tooley et al. (29).
[b] Trout et al. (32).

DNA was always detected while 0.1 fg was detected half of the time. Stochastic processes or sequence heterogeneity may limit amplification at 0.1 fg, since this represents <3 copies of O8.

In these reactions and those described below, the CA and MD laboratories intentionally used different sources of primers, buffers, nucleotides, *Taq* polymerase, thermal cyclers, and *P. infestans* DNA as a real-world test of the robustness of the assays. Some differences between locations were noted. For example, the O8 primers were slightly more sensitive in MD than CA. Sensitivities of the AE7 and ITS-based primers also varied between CA and MD, although neither were more sensitive than the O8 primers based on six side-by-side comparisons.

Specificity of PCR. The O8 and AE7 primers displayed adequate specificity based on amplifications using 1 ng of DNA from 33 species of *Phytophthora* (Table 2), including all infecting potato or tomato (9); other oomycetes, true fungi, and bacteria including common pathogens of potato and tomato (Table 3); and plants. Representative gels from CA are shown in Figure 3. Clear bands were generally detected only against *P. infestans* (lane 1), *P. mirabilis* (lane 26), and *P. phaseoli* (lane 27). Primers developed previously also failed to distinguish these species, which apparently evolved recently from a common ancestor (20,30). Such cross-reaction is largely an academic issue since the latter two species do not infect the same hosts as does *P. infestans*.

Faint or spurious bands were observed against a few *Phytophthora* spp. Weak bands of the size expected for *P. infestans* were sporadically obtained using the AE7 primers against *P. hibernalis* (lane 16) and *P. ilicis* (lane 18), although this has minor consequence since neither infects potato or tomato. Bands differing in size from the *P. infestans* amplicons were seen occasionally

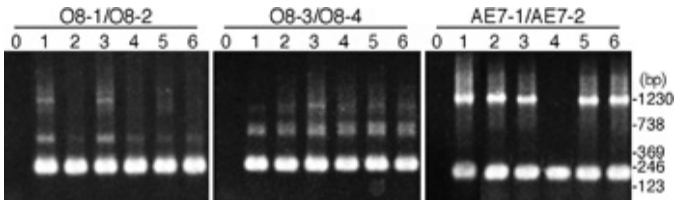

Fig. 1. Polymerase chain reaction using DNA from *Phytophthora infestans.* Reactions were executed using the indicated sets of primers with either no template (lane 0) or 1 ng of DNA from isolates 1484, 582, 6736, 1362, 6170, and 1163 (lanes 1 through 6). Indicated in the right margin are size standards from a 123-bp ladder. Reactions were performed in the California laboratory as described in text.

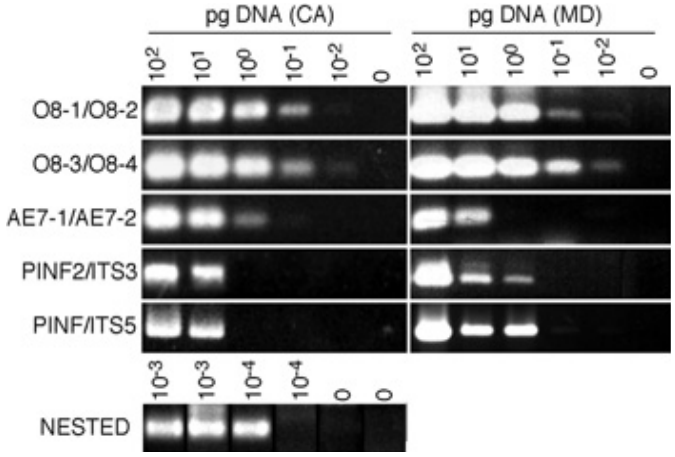

Fig. 2. Sensitivity of primers against *Phytophthora infestans.* The indicated primers were tested in the California and Maryland laboratories against DNA from isolates 618 and 127, respectively. Nested polymerase chain reaction was performed with 10^{-2} to 10^{-4} pg of DNA as a template; amplification was first performed (in duplicate) with primer set O8-1/O8-4, and then a 1:25 dilution of that reaction was amplified using the O8-3/O8-4 set. Data shown are representative of at least four replicates.

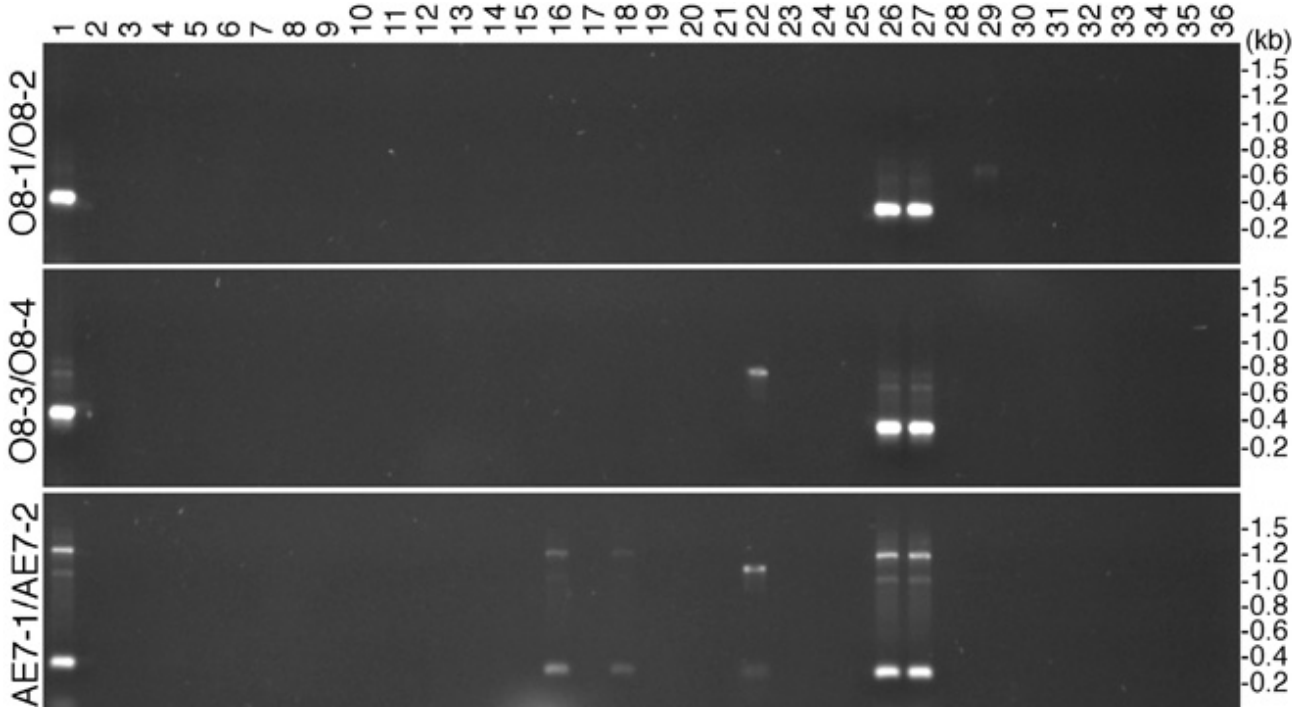

Fig. 3. Specificity of primers against *Phytophthora* and plant species. Amplifications were performed using 1 ng of DNA from species of *Phytophthora* known to be pathogenic on tomato or potato (lanes 1 through 9), species of *Phytophthora* not colonizing those plants (lanes 10 through 33), and tomato and potato (lanes 34 and 35). 1, *P. infestans*; 2, *P. cactorum*; 3, *P. capsici*; 4, *P. cinnamomi*; 5, *P. cryptogea*; 6, *P. dreschleri*; 7, *P. erythroseptica*; 8, *P. nicotianae*; 9, *P. palmivora*; 10, *P. boehmeria*; 11, *P. citricola*; 12, *P. citrophthora*; 13, *P. colocasiae*; 14, *P. fragariae*; 15, *P. heaveae*; 16, *P. hibernalis*; 17, *P. humicola*; 18, *P. ilicis*; 19, *P. inflata*; 20, *P. insolita*; 21, *P. iranica*; 22, *P. katsurae*; 23, *P. lateralis*; 24, *P. meadii*; 25, *P. megasperma*; 26, *P. mirabilis*; 27, *P. phaseoli*; 28, *P. porri*; 29, *P. quininea*; 30, *P. richardiae*; 31, *P. sojae*; 32, *P. tentaculata*; 33, *P. vignae*; 34, *L. esculentum* cv. New Yorker; 35, *S. demissum*; 36, no template. Size standards were determined by a 100-bp ladder. Data shown are from California laboratory.

(i.e., not in all replicates) using O8-1/O8-2 with *P. quininea* (lane 29), O8-3/O8-4 with *P. katsurae* (lane 22) and *P. capsici* (lane 3), and AE7-1/AE7-2 with *P. katsurae.*

The robustness of the specificity of the AE7 and O8 primer sets was tested over a range of conditions. Similar results were obtained whether amplification was performed at 0.6, 1.0, 1.5, or 2.0 mM Mg^{2+}; pH 8.2, 8.5, or 8.8; 40 or 65 mM salt; 0 or 0.1% Triton X-100; 0.2 or 1.0 µM of each primer; and 0.05 or 0.1 mM dNTPs.

Results between CA and MD varied only slightly. Discrepancies generally involved *Phytophthora* spp. that are classified in Group IV of the Waterhouse scheme, where *P. infestans* is also placed (33). This was examined in detail for the O8-3/O8-4 primers, where *P. ilicis* and *P. colocasiae* yielded weak bands in MD but none in CA (Fig. 4, bottom three groups of panels). The possibility that this reflected differences in quality, purity, or source isolate of the DNA was excluded by sharing DNA between CA and MD. The sporadic amplification of weak bands from *P. colocasiae* and *P. ilicis* has little practical significance since neither infects potato or tomato; the fact that pathogenic species such as *P. capsici, P. erythroseptica,* and *P. dreschleri* were negative at both sites (Fig. 4) is more noteworthy.

A second example of results varying between the laboratories was that in MD, but not CA, the O8-3/O8-4 primers often generated weak bands from potato (*S. tuberosum* and *S. demissum).* The potato bands were easily distinguished from the *P. infestans* amplicons by size, however. The minimum threshold for amplification, in terms of nanograms of template DNA, was 10^6 to 10^7 higher for potato than *P. infestans.* Based on exchanges of DNA between CA and MD, the ability to amplify bands from potato was shown to be location-specific. Such bands were only obtained in CA when unrealistically large amounts of template (100 ng) were employed. Raising annealing temperatures to 54°C improved specificity, but reduced sensitivity for *P. infestans* by twofold.

Since the O8 and AE7 primers behaved slightly differently in CA and MD, the specificities of ITS primers were also examined (Fig. 4, top four rows). The PINF2/ITS3 combination showed good specificity in both sites. The PINF/ITS5 set behaved similarly in CA and MD but 1 ng of DNA from potato yielded bands that were close in size to the *P. infestans* amplicon. That this resulted from using contaminated potato DNA was considered but excluded by several criteria, including obtaining DNA from other laboratories or tissue culture plantlets which should be devoid of *P. infestans.* Nevertheless, the PINF/ITS5 primers should still be useful for most diagnostic assays since no bands were detected using DNA from small amounts of tuber tissue, which is relatively low in potato DNA.

Development of amplification mimic. A mimic, or competitor, for the O8-3/O8-4 amplicon was developed as a control for diagnostic assays and a standard for quantitative PCR. Candidates were generated by low-stringency PCR against *E. coli* DNA; a 390-bp band with O8-3 and O8-4 at either end was selected and cloned into a plasmid. The minimum mass of template plasmid that reliably yielded this band was 0.2 fg, or 54 copies. Up to 5 fg did not obviously inhibit amplification of the native 260-bp band when mixed with 1 to 1,000 fg of *P. infestans* DNA (Fig. 5) or DNA from infected tubers (Fig. 6), supporting its utility as a control against false negatives in diagnostic PCR. Note that the ab-

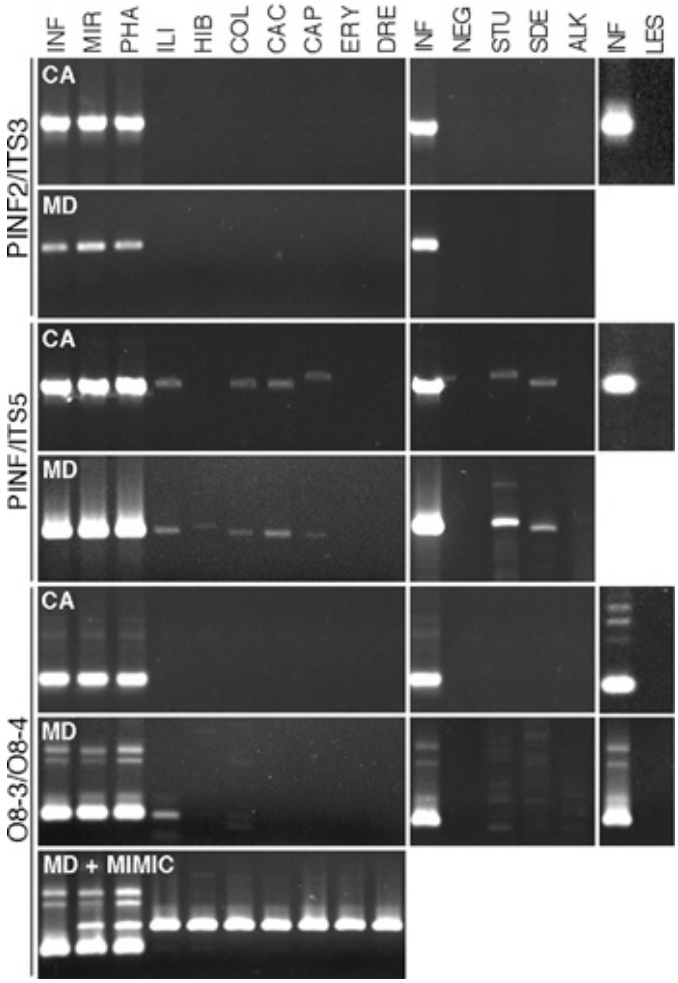

Fig. 4. Specificity of O8 and ITS primers in California (CA) and Maryland (MD) laboratories and effect of mimic. Polymerase chain reaction (PCR) was performed using PINF/ITS5, PINF2/ITS3, or O8-3/O8-4 as indicated; the source of the data (CA or MD) is labeled within each row. Left panel, PCR using 0.1 ng of DNA from *Phytophthora infestans* (INF), other members of Waterhouse group IV *(P. mirabilis* [MIR], *P. phaseoli* [PHA], *P. ilicis* [ILI], *P. hibernalis* [HIB], *P. colocasiae* [COL]), and selected potato or tomato pathogens (*P. cactorum* [CAC], *P. capsici* [CAP], *P. erythroseptica* [ERY], and *P. dreschleri* [DRE]). Middle panel, 1 ng DNA from *P. infestans* (INF), no template (NEG), 1 ng of DNA from *S. tuberosum* cv. Katadhin (STU); 1 ng DNA from *S. demissium* (SDE); DNA extracted from 1 mg tuber tissue using the NaOH method (ALK). Right panel, 1 ng DNA from *P. infestans* (INF) and *L. esculentum* cv. New Yorker (LES). In the bottom row, 5 fg of amplification mimic was included in reactions.

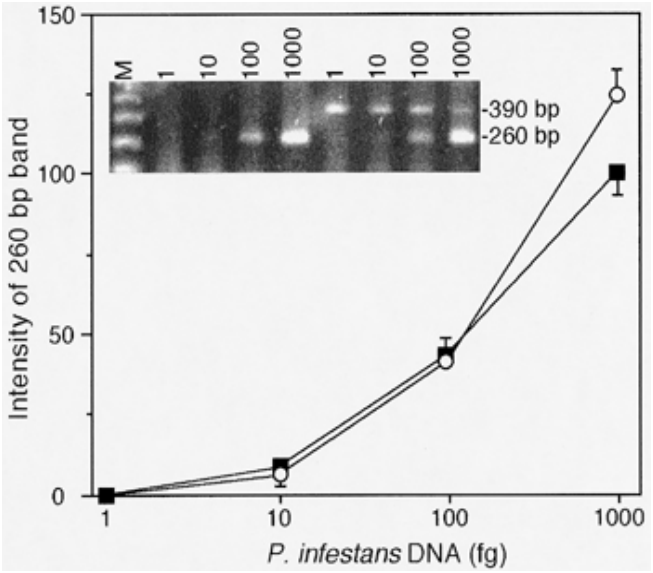

Fig. 5. Effect of mimic on sensitivity. A dilution series of *Phytophthora infestans* DNA (isolate 550) was subjected to PCR using primer set O8-3/O8-4 in the presence (■) or absence (○) of 5 fg of mimic plasmid. Band intensities were measured after electrophoresis of the reaction products in the presence of ethidium bromide. The 390 and 260-bp bands shown in the photograph correspond to mimic and native *P. infestans* amplicons, respectively. Lanes labeled 1 to 1,000 indicate the amount of *P. infestans* DNA per reaction (in fg), and lane M contains a 123-bp ladder.

sence of the mimic amplicon in most infected tubers is expected due to competition; the mimic serves as a positive control in otherwise negative samples. Attempts to generate a suitable O8-1/O8-2 mimic were unsuccessful.

The O8-3/O8-4 mimic exerted either beneficial or neutral effects on specificity. Inclusion of 5 fg of the mimic with DNA from the species listed in Table 2 yielded no bands not observed earlier, and weak bands previously noted in MD for *P. ilicis* and *P. colocasiae* became nearly imperceptible (Fig. 4, bottom panel). Spurious bands previously seen in CA (as from *P. katsurae)* did not amplify in the presence of the mimic, but amplification from *P. mirabilis* and *P. phaseoli* was unaffected.

The mimic also was validated as an appropriate competitor for quantitative PCR. This was shown by coamplifying 40 pg of *P. infestans* DNA with amounts of mimic plasmid that contained the number of copies of O8 in 4.8 to 1,400 pg of *P. infestans* DNA, as calculated based on the estimated copy number of the target sequence (15; Fig 7A). The $\log_{10}$ of the PCR product ratio (390-bp/260-bp bands) displayed a linear relationship versus $\log_{10}$[mimic] over >2 orders of magnitude (Fig. 7B). This confirms the mimic's suitability as a competitor for quantitative PCR (10).

Quantification of *P. infestans* in tomato. The growth of *P. infestans* during compatible and incompatible interactions on tomato leaflets was studied by quantitative PCR using O8-3 and O8-4, and the results were compared to assessments of lesion size (Fig. 8). This involved cultivars bearing resistant and susceptible alleles of resistance gene *Ph1* (New Yorker and Pieraline, respectively) challenged with isolate 618, which expresses the matching *Le1* avirulence gene (27). Differences between the two types of assays were apparent, which was anticipated since lesions largely result from plant response and not pathogen growth per se. For example, lesion area increased late on the resistant plant whereas DNA levels fell, which might reflect the degradation of DNA within dying hyphae. DNA levels in the compatible interaction also outpaced lesion area after day two, perhaps because lesion measurements understate the three-dimensional growth of the pathogen.

DISCUSSION

Several methods for diagnosing late blight have been reported, including PCR. Despite its advantages, PCR has limitations. For example, primers and reaction conditions conferring suitable sensitivity are required; high sensitivity is imperative for *P. infestans* due to the devastating nature of late blight and the need to diagnose young lesions. Specificity is required to avoid erroneous positives, and false negatives due to procedural errors or amplification inhibitors must be managed. Previous PCR methods for *P. infestans* did not address quantification, except for a recent study using expensive fluorescence PCR equipment (2), or false negatives. This study addresses the above issues by introducing new primers with enhanced sensitivity and good specificity, plus an amplification mimic to identify false negatives and enable quantification. Furthermore, the robustness of the assays was tested by replicating experiments in two locations using independent reagents.

The O8-based primers exhibited higher sensitivity than those previously reported, based on side-by-side multilaboratory comparisons with ITS primer sets and published values for others. This is likely due to the higher abundance of the O8 target, which is about 14,000 copies per nucleus. This compares to about 820 and 30 copies, respectively, of the ITS region (15) and the target used by Niepold and Schöber-Butin (22). All applications may not require the high sensitivity of the O8 primer sets, but such levels of sensitivity should engender increased accuracy when testing for *P. infestans* in samples that contain very young lesions, those pooled from large volumes of plant tissue, material containing amplification inhibitors, or specimens containing partially degraded DNA. The internal positive control represents another advance in PCR technology for *P. infestans.* The importance of internal standards in excluding false negatives is accepted in medical applications of PCR (5,28). Internal controls are only occasionally adopted for plant pathogens (14), which is inappro-

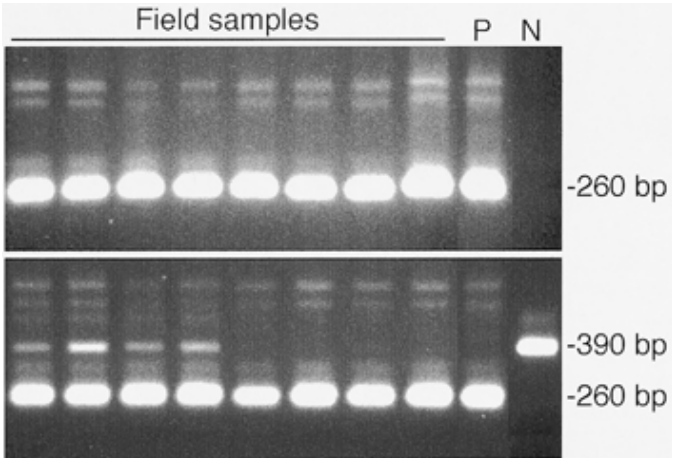

Fig. 6. Detection of *Phytophthora infestans* in tubers. Tubers (cv. Katahdin) showing symptoms of late blight were obtained from a field in Maine. DNA was extracted using the NaOH method and subjected to polymerase chain reaction using primer set O8-3/O8-4 without (top panel) or with (bottom) 5 fg of mimic plasmid. Lane P, positive control. Lane N, negative control.

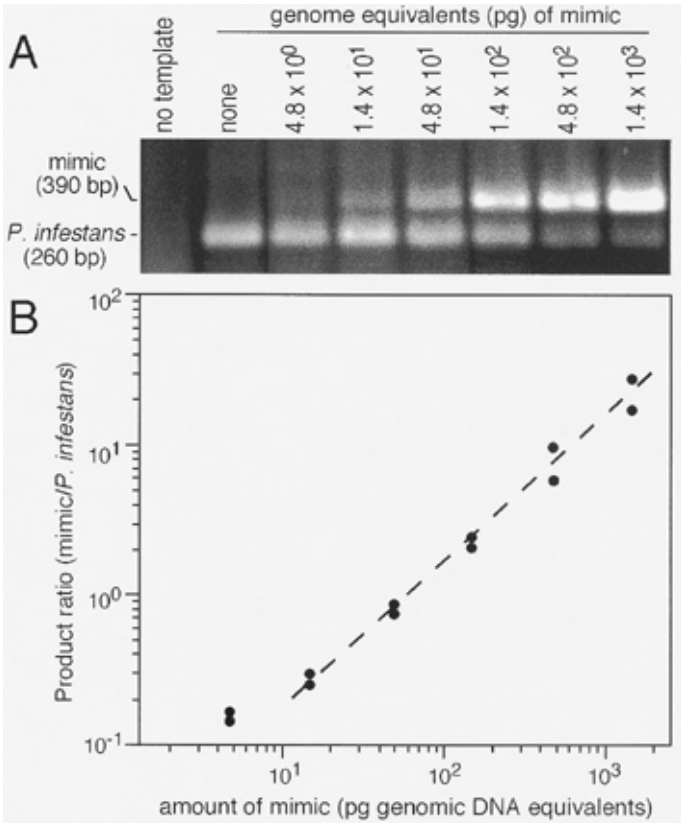

Fig. 7. Interaction between mimic and native amplicons during polymerase chain reaction. Genomic DNA of *Phytophthora infestans* (40 pg) and increasing amounts of the mimic plasmid (corresponding to the number of copies of O8 in 0 to 1.4×10^3 pg of *P. infestans* DNA) were subjected to PCR using primer set O8-3/O8-4, and reaction products were quantified after electrophoresis in the presence of ethidium bromide. Two replicates were measured. **A,** Gel of reaction products from one of the replicates. The leftmost reaction contained neither mimic nor *P. infestans* DNA. Indicated are the mimic and genomic amplicons (390 and 260 bp, respectively). **B,** Ratio of band intensities (390 bp/260 bp) versus amount of mimic. A 1:1 slope is represented by the dashed line.

priate considering that crude template preparations, which may contain inhibitors, are generally used; a mere 10% reduction in amplification per cycle may reduce sensitivity 40-fold after 35 cycles (0.9^{35}). Using minimal amounts of control template also contributes to quality control by ensuring that PCR is at peak efficiency. An internal coamplified control has advantages over parallel controls because only the former identifies random failures caused by mispipetting, inhibitors, bad wells in gels, and other factors.

The control amplicon also enabled the quantitative measurement of *P. infestans* by competitive PCR using a dilution series of mimic. Semi-quantitative data may also be obtained from a single reaction since the intensity of the mimic amplicon falls as the *P. infestans* target increases. Quantification should also be possible using the O8 primers in fluorescence PCR, although such technology may be too expensive for broad adoption.

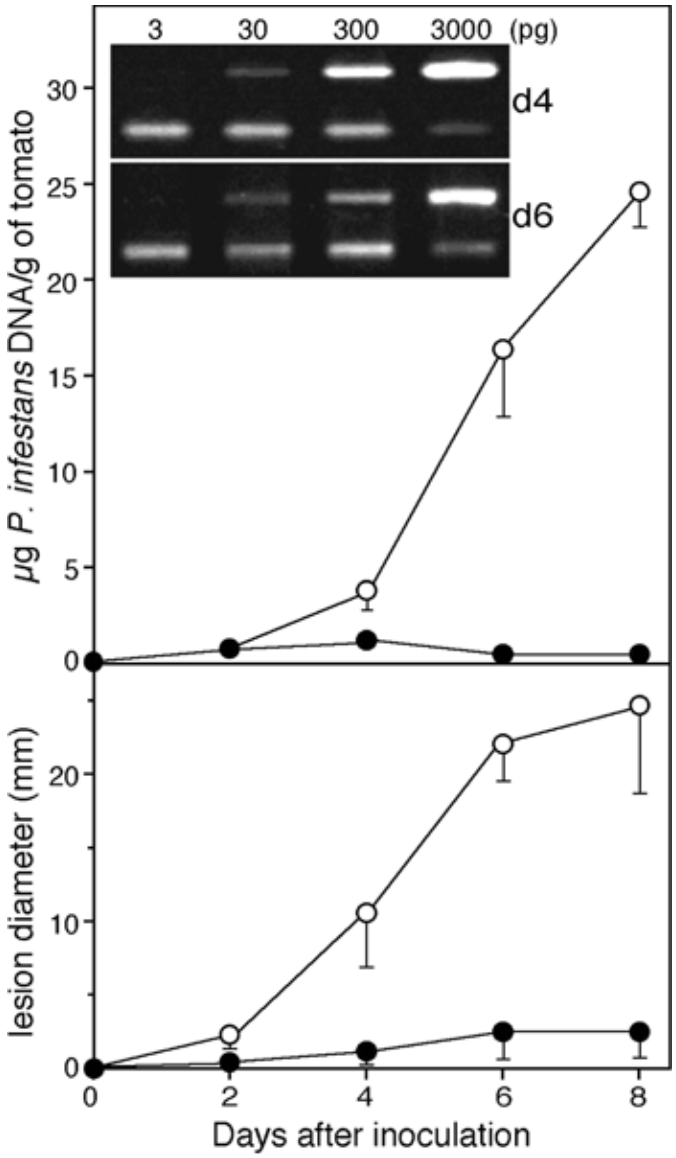

Fig. 8. Measurement of *Phytophthora infestans* in tomato leaflets by competitive polymerase chain reaction. Leaflets of resistant cv. New Yorker (●) and susceptible cv. Pieraline (○) were inoculated with isolate 618 and incubated at 15°C. DNA was extracted from two pools of eight leaflets per tomato genotype at each time-point. *P. infestans* DNA per gram of tomato tissue (top panel) was determined by measuring the intensity of mimic and *P. infestans* amplicons in each reaction, and then calculating the mimic concentration at which those bands would have had the same intensity; the resulting equivalence point (10) would indicate the concentration of *P. infestans* target DNA in the sample. Error bars represent variation between duplicate pools. The photograph shows representative assays at days 4 and 6 on cv. Pieraline, with mimic concentrations corresponding to 3, 30, 300, and 3,000 pg of *P. infestans* DNA. Average lesion diameters (± standard deviation) are shown in the bottom panel. Error bars within symbols are not shown.

An issue that was stressed in this paper is that PCR results between different laboratories may vary even when similar protocols are employed. Anecdotal reports of such phenomena are common yet rarely reach the scientific literature (23); the point is worth emphasizing considering the diverse experience of workers adopting PCR. For the O8 primers, deviations in sensitivity and specificity were minor and inconsequential. The major difference noted was that only in MD did weak bands sometimes amplify from potato, although these were readily distinguished from the *P. infestans* band and, arguably, might be beneficial as a positive control. In contrast, for the AE7 primers, major disparities in sensitivity were noted between CA and MD. Specificity also varied for ITS3/PINF in CA, MD, and in Trout et al. (32).

Explanations for differences between laboratories can be proposed. Reagents such as water, primer, nucleotides, and DNA polymerase can vary in quality. It might be practical to standardize such variables, but not others such as the thermal cycler; heating and cooling rates of different models vary substantially, for example, influencing the specificity of PCR (6,34). A point to be stressed is that uniformity in reagents and equipment is difficult to achieve in the real world. We consequently propose that authors of manuscripts describing diagnostic PCR assays should have a colleague in another laboratory test their assay. This is rarely the case outside of medical or forensic applications (35).

In response to conversations with J. Ristaino made after acceptance of the manuscript, we tried to address why the PINF/ITS5 primers exhibited inferior specificity in the current study compared to that noted by Trout et al. (32). We therefore performed a side-by-side comparison using DNA from potato, *P. capsici*, and *P. infestans* with the PINF/ITS5 primers as described in this paper (using thin-wall tubes, 25-µl reactions with no mineral oil overlays, and a fast-cycling thermal cycler with 30, 30, and 60 s denaturation, annealing, and extension times, respectively) and by Trout et al. (thick-wall tubes, 50-µl reactions with mineral oil, and longer cycling times in a different thermal cycler). Using the latter conditions, nonspecific bands were generally not obtained against potato or *P. capsici*. However, this came at the cost of a 10-fold reduction in the sensitivity of detection of *P. infestans* DNA. This supports the point made previously in Discussion that primer sets can exhibit divergent behavior in different laboratories due to variables that may be unanticipated or impractical to control.

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