Identification and Quantification of Pathogenic *Pythium* spp. from Soils in Eastern Washington Using Real-Time Polymerase Chain Reaction

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ABSTRACT

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Traditional methods of quantifying *Pythium* spp. rely on the use of selective media and dilution plating. However, high variability is inherent in this type of enumeration and counts may not be representative of the pathogenic population of *Pythium* spp. Variable regions of the internal transcribed spacer of the rDNA were used to design species-specific primers for detection and quantification of nine *Pythium* spp. from soils in eastern Washington. Primer pairs were designed for *Pythium abappressorium*, *P. attrantheridium*, *P. heterothallicum*, *P. irregulare* group I,

P. irregulare group IV, *P. paroecandrum*, *P. rostratifingens*, *P. sylvaticum*, and *P. ultimum* and used with real-time polymerase chain reaction. Standard curves were generated for each of the species using SYBR Green I fluorescent dye for detection of amplification. Seventy-seven isolates of *Pythium* were screened to confirm specificity of each primer set. DNA was extracted from soil and standard curves were generated for *P. irregulare* group I, *P. irregulare* group IV, and *P. ultimum* to correlate populations of each species in the soil with quantities of DNA amplified from the same soil. Examination of raw field soils revealed results similar to those observed in previous studies. This new technique for the quantification of *Pythium* spp. is rapid and accurate, and will be a useful tool in the future study of these pathogenic *Pythium* spp.

Pythium root rot is a chronic disease of wheat and barley in eastern Washington. More than 10 species of *Pythium* were implicated in causing root rot and yield loss of wheat and barley in Washington (8,20,21) and numerous species recently were recovered from field soils cropped to wheat (42). These species possess varying degrees of virulence, and the population dynamics of these pathogens is complex. In a recent survey of *Pythium* spp. throughout eastern Washington, up to six species were recovered from a single soil sample and many different species combinations were identified (42).

Diagnosis of *Pythium* root rot in the field is challenging due to similarities to symptoms of other seedling diseases. In addition, root symptoms are not readily evident, because obvious lesions do not form on the roots. The primary symptom is reduced seedling vigor, which may be manifested as reduced emergence, plant stunting, reduced first leaf length, and destruction of fine roots and root hairs. However, damage usually is limited to stunting of the plant, and these pathogens rarely kill the plant (13).

Traditional methods of studying the population dynamics of *Pythium* spp. have involved dilution plating onto a selective medium (1,11,33) and the observation of morphological features to identify individual isolates to species (48). However, these techniques pose some serious constraints to studying this group of pathogens; morphological identification is time consuming and difficult, and some species are asexual or heterothallic, making identification without sexual structures difficult or impossible. The results of dilution plating to determine populations of *Pythium*

spp. also may be misleading due to the presence of nonpathogenic species of *Pythium* or pathogenic species that grow too slowly to be counted. In a collection of *Pythium* spp. from 80 wheat fields from eastern Washington, Paulitz and Adams (42) found that 30.2% of the isolates were nonpathogenic *P. oligandrum*. More recently, molecular approaches were used to identify *Pythium* spp., including the use of restriction fragment length polymorphisms (9,10,51), hybridization probes (23,26,27), polymerase chain reaction (PCR) (49), PCR enzyme-linked immunosorbent assay (4), and sequencing (25,29,30,42).

Many of these molecular studies with *Pythium* spp. have focused on the use of the internal transcribed spacer (ITS) sequence from the nuclear ribosomal DNA for species differentiation, although the cytochrome oxidase II gene, encoded by the mitochondria, also has been used for phylogenetic comparisons (29). The ITS regions within *Pythium* spp. are conserved within a species but variable between species, making differentiation of species using these spacer regions possible (9,10,25,30). The use of ITS sequence data has been a valuable tool along with morphological features to differentiate several new species of *Pythium* over the past several years (2,34–41,43). Morphological differences are sometimes subtle or overlooked by less-experienced mycologists when working with these pathogens. Recently, sequence data of ITS1 and ITS2 was used to confirm the identity of *Pythium* spp. collected in eastern Washington (42).

Although previously used molecular techniques were very sensitive and capable of separating many species of *Pythium*, the techniques were not quantitative or designed for identification directly from a soil or plant sample. A solution to this is real-time PCR for specific amplification and quantification of various species. Real-time PCR quantifies DNA by using a fluorescent dye or probe to detect the product. Monitoring fluorescence during the cycling (52) allows the start of the logarithmic phase of amplification to be identified in order to calculate the initial

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starting concentration of target DNA (6). Specificity can be established by running a melting curve at the end of cycling. Each product formed will have a distinct melting profile based on the length and base composition of the product, providing the means to confirm that a specific amplification occurred. Real-time PCR assays previously were developed for other members of the stramenopila (7,19,47) and several plant-pathogenic fungi (6,14, 15,17,24,46).

The objectives of this study were to develop primers for the ITS region to use with real-time PCR that would differentiate nine of the most common pathogenic species of *Pythium* from soils in Washington, and to develop an assay using soil DNA extraction techniques and real-time PCR to identify and quantify *Pythium* spp. in soil. This was accomplished by identifying species-specific primers for nine species of *Pythium* and using real-time PCR in conjunction with SYBR Green I dye for detection of the product. Standard curves were generated for each species and a soil extraction protocol was developed by modification of a commercially available soil DNA extraction kit.

MATERIALS AND METHODS

Maintenance of *Pythium* spp. cultures. Seventy-seven isolates of *Pythium* representing the nine most commonly isolated pathogenic species found in eastern Washington (42) were used in this study. Working cultures were maintained on potato dextrose agar (PDA) (Difco Laboratories, Sparks, MD) and isolates were stored at 4°C on PDA slants. A collection of fungal isolates was used for confirming the specificity of the *Pythium* primers developed in this study (Table 1). These cultures all were maintained on PDA, with the exception of *Phytophthora infestans*, which was cultured on corn meal agar.

DNA extraction and sequencing. All isolates of *Pythium* spp. used for sequencing are listed in Table 1. Each isolate of *Pythium* was cultured in 15 ml of potato dextrose broth in a petri dish (100 by 15 mm) at room temperature until most of the plate was covered with mycelium (5 to 14 days, depending on the isolate). Mycelial mats were washed in distilled water and blotted dry, and

DNA was extracted using the FastDNA Kit (Qbiogene, Carlsbad, CA) along with a FastPrep FP120 cell disrupter (Qbiogene) as described by Paulitz and Adams (42). The ITS regions (ITS1 and ITS2) as well as the 5.8S gene of the rDNA were amplified using the universal eukaryotic primers UN-UP18S42 (5'-CGTAACA-AGGTTTCGTTAGGTGAAC-3') and UN-LO28S576B (5'-GT-TTCTTTTCCCTCCGCTTATTAATATG-3') (5) to produce DNA template for sequencing. Amplification of DNA was performed using a PTC-200 Peltier thermal cycler (MJ Research, Reno, NV) in a 20-µl reaction consisting of 16.8 µl of nanopure water, 2.5 µl of 10× buffer, 1.9 mM MgCl₂, 0.2 mM dNTPs, 10 pmol of each primer, 0.2 µl of *Taq* DNA Polymerase (1 unit), and 0.5 µl of DNA template. The *Taq* Polymerase, 10× buffer, and MgCl₂ were obtained from Promega Corporation (Madison, WI). The following program was used to amplify the DNA: 3 min at 94°C (1 cycle); 45 s at 92°C, 45 s at 60°C, and 60 s at 72°C (30 cycles); and 10 min at 72°C (1 cycle).

Prior to sequencing, remaining dNTPs and primers were removed from the PCR product by treating 5 µl of the product with 2 µl of ExoSAP-IT (USB Corporation, Cleveland, OH). The mixture was incubated at 37°C for 30 min and the enzymes were inactivated by incubation at 80°C for 15 min. Sequencing reactions were completed for both strands of the ITS regions and the 5.8S rDNA using the universal eukaryotic forward and reverse primers listed above, along with two internal primers located in the 5.8S gene. OOM-LO5.8S47B (5'-CGCATTACGTATCGCAG-TTCGCAG-3') is a reverse primer located at the 5' end of the 5.8S and amplified ITS1, whereas OOM-UP5.8S01 (5'-CAACT-TTCAGCAGTGGATGTCT-3') is a forward primer located at the 3' end of the 5.8S gene to amplify ITS2 (32). The sequencing reactions were set up using a 10-µl total volume that consisted of 4 µl of ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA), 4 pmol of primer, and 5 µl of ExoSAP-IT product. The following program was used to amplify the DNA: 2 min at 94°C (1 cycle); and 2 min at 94°C, 1 min at 50°C, and 1.5 min at 60°C (24 cycles). Reactions were run through a Sephadex G-50 column using 1 ml of a Sephadex suspension in a mini-column. The columns were pre-

TABLE 1. Isolates of *Pythium* spp. and other fungi used in this study

Species	Isolate ^a	Site of isolation ^b	Origin (county, city) ^c	GenBank accession no.	Source ^d
<i>Pythium abappressorium</i>	020162	Wheat	Adams, Ritzville	DQ091294	T. Paulitz
<i>P. attrantheridium</i>	020169	Barley	Lincoln, Davenport	DQ091296	T. Paulitz
<i>P. heterothallicum</i>	090014	Barley	Kootenai, Rockford	DQ091298	T. Paulitz
<i>P. irregulare</i> group I	0900101	Spring wheat	Benewah, Tensed	DQ091295	T. Paulitz
<i>P. irregulare</i> group IV	020155	Spring wheat	Spokane, Latah	DQ091299	T. Paulitz
<i>P. paroecandrum</i>	090031	Lentil	Whitman, Palouse	DQ091297	T. Paulitz
<i>P. rostratifingens</i>	020172	Spring wheat	Columbia, Dayton	DQ091300	T. Paulitz
<i>P. sylvaticum</i>	0900115	Lentil	Whitman, Pullman	DQ091301	T. Paulitz
<i>P. ultimum</i>	030141	—	Kootenai, Rockford	DQ091302	T. Paulitz
<i>Aphanomyces euteiches</i>	Ae02A9	Pea	Eastern Washington	—	N. Grünwald
<i>Bipolaris sorokiniana</i>	BS15e-99	Winter wheat	Eastern Washington	—	K. Schroeder
<i>Fusarium culmorum</i>	F070366	Wheat	Whitman, Pullman	—	T. Paulitz
<i>F. pseudograminearum</i>	006-13	Winter wheat	Wasco, Unknown	—	R. Smiley
<i>Gaeumannomyces graminis</i> var. <i>tritici</i>	R3-111a-1	Wheat	Grant, Moses Lake	—	D. Weller
<i>Mortierella hyalina</i>	P2c3	Winter wheat	Whitman, Pullman	—	K. Schroeder
<i>Phytophthora infestans</i>	W03GE	Potato	Grant, George	—	D. Johnson
<i>Rhizoctonia oryzae</i>	Rh070332	Wheat	Adams, Lind	—	T. Paulitz
<i>Rhizoctonia solani</i>	Rh100279	Chickpea	Walla Walla, Walla Walla	—	T. Paulitz
<i>Trichoderma polysporum</i>	P1cc9	Winter wheat	Whitman, Pullman	—	K. Schroeder
<i>T. viride</i>	W3b4	Winter wheat	Whitman, Pullman	—	K. Schroeder

^a All *Pythium* spp. were identified by morphology and identity was confirmed by sequence analysis of the internal transcribed spacer (ITS)1 using the database of C. A. Lévesque, Agriculture and Agri-Food Canada, Ottawa, Canada.

^b *Pythium* and *Rhizoctonia* isolates were recovered from the soil of the crop listed. All other genera were recovered from plant material or rhizosphere soil. Isolates with a dash (—) were isolated from agricultural fields that were either fallow or harvested prior to sampling.

^c All isolates used in this experiment were from various counties and cities in Washington, with the exception of those from Benewah and Kootenai county in Idaho and the *F. pseudograminearum* isolate from Oregon.

^d Isolates were obtained from T. C. Paulitz, United States Department of Agriculture—Agricultural Research Service (USDA-ARS), Pullman, WA; N. Grünwald, USDA, Corvallis, OR; K. L. Schroeder, Washington State University, Pullman; R. W. Smiley, Oregon State University, Pendleton; D. M. Weller, USDA-ARS, Pullman, WA; and D. A. Johnson, Washington State University, Pullman.

spun at $750 \times g$ for 2 min, and then the sequencing reaction was brought up to 20 μl by adding nanopure water, placed onto the top of the spin column, and spun for an additional 2.5 min at $750 \times g$. The sequence reactions were run on an Applied Biosystems sequencer (model 377; Applied Biosystems). At least two complete sequences were obtained for each region of the ITS1, 5.8S, and ITS2 sequence. The resulting sequences were assembled using SeqMan II (version 5.00; DNASTAR, Madison, WI), aligned using ClustalW version 1.8, and box shaded using GeneDoc (version 2.6.002) (Pittsburgh Supercomputing Center, Pittsburgh, PA).

Primer design and preliminary screen for specificity. Primer pairs were selected for *P. abapressorium* Paulitz & Mazzola, *P. attrantheridium* Allain-Boulé & Lévesque, *P. heterothallicum* W. A. Campb. & F. F. Hendrix, *P. irregulare* Buisman (groups I and IV), *P. paroecandrum* Drechsler, *P. rostratiformis* de Cock and Lévesque, *P. sylvaticum* W. A. Campb. & F. F. Hendrix, and *P. ultimum* Trow using the variable regions of ITS1 and ITS2. Each primer was designed with an annealing temperature of ≈ 68 to 70°C and analyzed for dimer or hairpin loop structures using GeneRunner (version 3.05; Hastings Software, Inc., Hudson, NY). A primer sequence database was generated in Primer-Select (version 5.00; DNASTAR) and a preliminary screen was conducted using each *Pythium* sequence and examining for cross specificity. Primer pairs that would generate a PCR product of 100 to 400 bp were selected for further study and initially screened for dimer formation using Oligo (version 6.65; Molecular Biology Insights, Cascade, CO). Primer pairs were optimized for temperature and MgCl_2 concentration using an iCycler iQ gradient thermal cycler (Bio-Rad, Hercules, CA). Each 10- μl PCR reaction consisted of 9.8 μl of nanopure water, 1.5 μl of 10 $\times$ buffer, 1.5 to 4.0 mM MgCl_2 , 0.2 mM dNTPs, 6 pmol of each primer, 0.15 μl (0.75 units) of *Taq* DNA Polymerase, and 1 μl of DNA template. The following program was used to amplify the DNA: 4 min at 94°C (1 cycle); 45 s at 92°C , 45 s at 50 to 70°C , and 60 s at 72°C (30 cycles); and 7 min at 72°C . PCR products were separated on a 2% agarose gel stained with ethidium bromide.

An isolate of *Pythium* representing each of the nine species was selected for a preliminary test for specificity (Table 1). Those primers that amplified only the species for which the primers were designed were selected for further screening of the entire collection of 77 isolates. Primer sets that resulted in any non-specific amplification were discarded.

Generation of standard curves. A standard curve was generated individually for each primer set, using the nine *Pythium* spp. isolates (Table 1) as template. The quantity of DNA for each isolate was determined in a 200- μl volume in 96-well plates using Hoechst 33258 dye and a Safire fluorescence plate reader (TECAN Research, Triangle Park, NC). The DNA concentrations for each isolate were standardized to a final concentration of ≈ 5 ng/ μl . A set of dilutions was completed with 10-fold reductions in DNA concentrations from 5 ng/ μl to 5 fg/ μl . A 20- μl PCR was set up in borosilicate glass capillaries in triplicate for each DNA concentration. The mix consisted of 12.8 μl of nanopure water, 2.0 μl of LightCycler FastStart DNA Master SYBR Green I (Roche Applied Science, Indianapolis, IN), 4 mM MgCl_2 , 8 pmol of each primer, and 2 μl of template DNA. Real-time PCR was conducted on a Roche LightCycler (Roche Applied Science) using the program 10 min at 95°C (1 cycle); and 10 s at 95°C , 5 s at 70°C , and 10 s at 72°C (50 cycles). A melting curve of the product was generated by ramping the temperature to 95°C for 0 s, back to 70°C for 15 s, and then incremental increases of $0.1^\circ\text{C}/\text{s}$ up to 95°C with continuous measurement of fluorescence. The resulting data were analyzed with the LightCycler Software version 3, using the arithmetic baseline adjustment and second derivative maximum analysis. The data was used to generate standard curves and the standard deviation of each DNA concen-

tration was calculated and plotted to estimate variability across a range of DNA quantities.

Evaluation of primer specificity using real-time PCR and in silico analysis. Each primer set was screened for specificity using the collection of 77 *Pythium* isolates and other genera (Table 1) by amplification using real-time PCR and the protocol listed above. The DNA concentrations for each isolate were standardized to 1.0 to 1.5 ng/ μl , and 2 μl of DNA was included in each reaction for a final concentration of 2 to 3 ng per reaction. For each primer set, the appropriate positive control was included at 1 ng per reaction, and water was used as the negative control.

Primer specificity also was evaluated by calculating the primer-target duplex stabilities (ΔG values) using the nearest neighbor model of PrimerSelect 5.00 at default scoring settings of 50 mM salt, 25°C , and $0.76 \text{ kcal mol}^{-1} \text{ bp}^{-1}$ average stability cut-off. Duplex stability values were obtained for the specific target sequences used to design the primers and 18 additional *Pythium* ITS sequences from GenBank that represented all nine target *Pythium* spp. To evaluate the potential of each primer to form hybrids with ITS sequences from nontarget *Pythium* spp., hybrid stability values were obtained in silico using 64 GenBank ITS sequences representing 28 nontarget *Pythium* spp. These species included *P. aphanidermatum*, *P. carolinianum*, *P. cylindrosporum*, *P. cystogenes*, *P. debaryanum*, *P. echinulatum*, *P. grandisporangium*, *P. hydnosporum*, *P. insidiosum*, *P. intermedium*, *P. kunmingense*, *P. litoralis*, *P. macrosporum*, *P. mamillatum*, *P. mastophorum*, *P. middletonii*, *P. minus*, *P. monospermum*, *P. myriotylum*, *P. oligandrum*, *P. pachycaule*, *P. pleroticum*, *P. rostratum*, *P. spinosum*, *P. splendens*, *P. torulosum*, *P. ultimum* var. *sporangiiferum*, *P. undulatum*, and *P. violae*.

Real-time PCR amplification from artificially infested and natural soils. Soils were artificially infested with *Pythium* spp. to evaluate the ability to extract and subsequently amplify *Pythium* DNA from soil. Ground oatmeal (Old Fashioned Style Quaker Oats; The Quaker Oats Company, Chicago) was added at 1% (wt/wt) to a Ritzville coarse silty loam soil from Lind, WA that was air dried and sieved through a 2-mm sieve. Water was added to the soil at 10% (wt/wt) and 70 g of the soil mixture was placed into 140-ml-volume jars with "B-cap" style Magenta lids. The soil was autoclaved on two consecutive days for 45 min each. One day prior to infesting the soils, 10 ml of water was added to each jar and allowed to infiltrate overnight. Three agar plugs (≈ 4 mm in diameter) from a 4- to 5-day culture were placed into each jar, and jars were shaken vigorously to mix the inoculum and disperse the moistened soil throughout the jar. Jars were incubated for 3 to 6 months prior to use. Populations of *Pythium* spp. within each jar were determined by serial dilution of 1 g of infested soil in water and plating onto *Pythium* selective medium (PSM) (33), using 2% water agar rather than cornmeal agar (2% water agar with pentachloronitrobenzene at 100 $\mu\text{g}/\text{ml}$, pimarinic acid at 0.25 $\mu\text{g}/\text{ml}$, rose bengal at 10 $\mu\text{g}/\text{ml}$, and rifampicin at 50 $\mu\text{g}/\text{ml}$).

A standard curve was generated to relate the population of *Pythium* spp. in soil as determined by dilution plating with the quantity of DNA amplified by real-time PCR. *P. irregulare* group I isolates 0900101 (Tensed, ID), 110305 (Garfield, WA), and 110306 (Garfield, WA); *P. irregulare* group IV isolates 020117 (Palouse, WA), 020147 (Palouse, WA), and 020155 (Latah, WA); and *P. ultimum* isolates 030141 (Rockford, WA), 10118 (Dusty, WA), and 10145 (Davenport, WA) were used to generate standard curves. *Pythium*-infested soils were diluted into a pasteurized Thatuna silt loam from the Spillman Agronomy Farm (Pullman, WA) to obtain final concentrations of 0, 10, 50, 100, 500, and 1,000 propagules per gram (ppg). Pasteurization was accomplished using a steam cart (Seibring Mfg., George, IA) at 60°C for 30 min. Total DNA was extracted from the soil samples using the UltraClean Soil DNA Kit (MO BIO Laboratories, Solana Beach, CA). From each soil dilution, three separate 500-mg samples of soil were processed with the DNA extraction kit using

the alternative protocol for maximum yields. The cell disruption step was modified, using the FastPrep FP120 at a speed of 5.0 for 45 s in place of vortexing for 10 min. The final volume from the DNA extraction procedure was 50 μ l. Real-time PCR reactions were conducted in a total volume of 20 μ l, using 2 μ l of undiluted DNA from the soil extractions. The reaction mix and PCR program were the same as described above. The resulting DNA quantification from real-time PCR was multiplied by 50 to obtain the concentration of DNA per gram of soil and plotted against the number of propagules per gram of soil as determined by dilution plating.

DNA extraction and real-time PCR amplification also was conducted using 500 mg of samples removed directly from the field. Soils were collected from five locations on 16 September 2004 following periodic rainfall occurring from 12 to 15 September 2004. These locations included a fallow field and a field cropped to barley north of Garfield, WA; a fallow field and a field cropped to winter wheat at the Cunningham Agronomy Farm northeast of Pullman, WA; and a field cropped to spring wheat at the Agricultural Research Service (ARS) Palouse Conservation Farm north of Pullman, WA. DNA extractions and real-time PCR were repeated after air drying a portion of each sample for 3 days. Total DNA was extracted from wet and dry soils using the protocol described above and real-time PCR was conducted as described previously. For this experiment, each of the nine primer pairs was tested on each soil sample. The populations estimated by real-time PCR were compared with results using traditional techniques of plating onto selective media. The total population of *Pythium* spp. in the soil was estimated by serial dilution of 1 g of soil in water and dilution plating onto PSM.

To rule out the presence of PCR inhibitors in the soil, purified DNA from *P. irregulare* group I, *P. irregulare* group IV, and *P. ultimum* was used to spike soil extracts in order to compare efficiencies. Extracts were made by using the UltraClean Soil DNA Kit as described above. Soils used in this assay were obtained from the Cunningham Farm, Garfield and the ARS Palouse Conservation Farm. A dilution series was made to test DNA concentrations at 10-fold increments from 0.01 to 100 pg. Real-time PCR was conducted as described previously and data plotted to obtain efficiencies. Mean comparisons of the efficiencies were conducted using Fisher's least significant difference (LSD) at $P = 0.05$. Analysis was performed using SAS (version 8.2; SAS Institute Inc., Cary, NC).

Confirmation of species identified in natural field soils. The identity of species detected and identified by real-time PCR were confirmed using traditional techniques for isolation and microscopic identification, and soil extracts also were processed using macroarray analysis (45). To determine the relative composition of *Pythium* spp. by traditional microscopy, 10 g of soil from each sample were placed into a petri dish and flooded with 25 ml of sterile distilled water. Fresh grass blades were placed onto the surface of the water and incubated for 24 h at room temperature ($\approx 21^\circ\text{C}$). The grass blades were transferred to PSM to select for *Pythium* spp., subcultured onto 2% water agar to obtain single isolates, and transferred to PDA for maintenance of the isolate. Agar plugs (≈ 4 mm in diameter) were transferred to a petri dish with ≈ 25 ml of sterilized pond water containing dried, sterilized grass blades. After 4 to 6 days of incubation at room temperature, the grass blades were examined for the presence of sporangia and oospores to identify isolates to species, using the taxonomic keys of Van der Plaats-Niterink (47).

A separate soil sample was collected from all three field locations in October 2005 for macroarray analysis. DNA was extracted from each soil and detection of *Pythium* spp. by DNA macroarray was accomplished using the protocols for PCR amplification, digoxigenin-labeling, and detection as described by Tambong et al. (45). Universal ITS primers for eukaryotes, UN-UP18S42 (forward, 5'-CGTAACAAGGTTTCCGTAGGTGAAC-

3') and OOM-LO28S345H (reverse, 5'-ACTTGTTTCGCTATC-GGTCTCGCA-3'), were used to produce labeled PCR amplicons. Hybridizations were done overnight and digoxigenin detected by chemiluminescence according to the protocol from the manufacturer by using anti-Digoxigenin alkaline phosphatase conjugate and the chemiluminescent substrate CDP-Star (Roche Diagnostics GmbH, Mannheim, Germany). Hybridizations were done twice to confirm results and specificity of the oligonucleotides.

RESULTS

DNA sequencing and primer design. A complete sequence of the ITS1, 5.8S, and ITS2 were obtained for each *Pythium* sp. listed in Table 1. Upon examining variable regions of the sequence, a series of forward and reverse primers were selected manually, using GeneRunner to eliminate primers with obvious dimer or hairpin loop problems. Between 2 and 14 primer sets initially were selected for each species of *Pythium*. A range of MgCl_2 concentrations was tested for each primer set; however, all primers functioned equally well at all concentrations tested (1.5 to 4.0 mM). Using a temperature gradient from 50 to 70°C , a number of primers were found to form substantial amounts of dimer at lower temperatures. Primer sets that consistently amplified at an annealing temperature of 70°C were used for further testing. A preliminary screen for specificity was conducted using traditional and real-time PCR and a species-specific primer pair was identified for each *Pythium* sp. that formed minimal or no dimers and resulted in no nonspecific amplification (Table 2). The primer pairs selected produce a product size of 277 to 414 bp and each set produced a characteristic melting peak.

Standard curves for real-time PCR. A single primer pair for each *Pythium* sp. was selected and standard curves were generated for each primer set using a range of DNA from 10 fg to 10 ng. Standard curves were obtained with very reproducible amplification and very high correlation values for each primer pair (Fig. 1). The slopes were very similar for each species, resulting in amplification efficiencies ranging from 80% for *P. paroecandrum* to 92% for *P. attrantheridium*. The y intercept (1 pg of DNA) for each species ranged from 24.74 cycles for *P. ultimum* to 28.41 for *P. paroecandrum*. The standard deviation of the log DNA concentration, calculated in generating the standard curves, varied from 0.004 to 0.096 (average = 0.033) at DNA concentrations of 0.1 to 10,000 pg (Fig. 2). However, at DNA concentrations < 0.1 pg of DNA (cycle threshold [C_t] value ≈ 32 to 36), the standard deviation ranged from 0.026 to 0.470 (average = 0.147).

Evaluation of primer specificity using real-time PCR and in silico analysis. Each set of primers was examined for specificity using the collection of 77 isolates of *Pythium* spp., with 2 to 14 isolates representing each of the species of *Pythium*. Due to the capacity of the LightCycler (32 samples), these isolates were divided randomly into three groups, with three separate runs necessary to test each primer pair. Every combination of *Pythium* isolate-primer set was tested. In every case, only those species for which the primer set was designed were amplified using real-time PCR (Table 3). A positive amplification was characterized by a C_t value in the range of 11.5 to 16.6 with a characteristic melting profile, and a negative amplification was characterized by a C_t value in excess of 29.0 as well as a melting profile indicative of nonspecific amplification. No nonspecific amplification was detected with any of the primer sets. Additional genera of fungi and oomycetes listed in Table 1 that either are found in the soils of agricultural production areas in eastern Washington or with similar phylogenetic relation to *Pythium* spp. were screened with each set of primers. In every case, the primers did not amplify target DNA from the nonspecific genera and, when nonspecific amplification did occur, the measured DNA concentration was below 1 fg and atypical melting peaks were generated.

The stability (ΔG values) of primer-target duplexes ranged from $-47.3 \text{ kcal mol}^{-1}$ for the *P. rostratifingens* primer ROS4F to $-39.8 \text{ kcal mol}^{-1}$ for the *P. heterothallicum* primer HET4F, with an average of $-43.3 \text{ kcal mol}^{-1}$ for all species-specific primers. Because these values were obtained for strains from Japan, China, France, and Australia, we expect that our primers will be useful for a wide geographical range of *Pythium* spp. In contrast, ΔG values for most of the nontarget (nonspecific *Pythium* spp.) hybrids were either below the default stability cut-off or greater than $-20 \text{ kcal mol}^{-1}$. These relatively high values are not correlated with significant or specific amplification under the conditions of our real-time PCR. Primers ABA1bF, IRR3cF, PAR2F or PAR3R (but not both), and SYL1F displayed hybrid stability values of less than $-32 \text{ kcal mol}^{-1}$ when complexed in silico with one or more of the species *P. cylindrosporum*, *P. debaryanum*, *P. insidiosum*, *P. intermedium*, *P. macrosporum*, *P. mamillatum*, *P. spinosum*, *P. torulosum*, *P. ultimum* var. *sporangiiferum*, and *P. violae*. Primer ATT2R also formed a stable hybrid with the *P. intermedium* ITS sequence ($\Delta G = -37.9$). Because only one primer of a pair formed a hybrid of $-32 \text{ kcal mol}^{-1}$ or less, significant amplification of nontarget DNA is not expected. However, amplification of *P. sylvaticum* DNA with PAR3R (and SYL1F) precludes multiplexing assays for *P. sylvaticum* and *P. paroecandrum*.

Real-time PCR amplification from artificially infested and natural soils. *Pythium* DNA was extracted successfully from artificially infested soil samples. In addition, the modification of the extraction protocol using the FastPrep FP120 was found to be an effective means of disruption. Soils were serially diluted to obtain populations densities that were representative of soils in eastern Washington and DNA was extracted from each dilution. Efficiency of DNA extraction and amplification with real-time PCR using primers specific for each species resulted in a proportional increase in DNA quantification across the entire range of *Pythium* populations examined, with correlations in excess of 0.909 for each species examined (Fig. 3). Using populations of *Pythium* spp. from ≈ 10 to 1,000 ppg, C_i values ranged from 31.29 to 24.41 for *P. irregulare* group I, 32.68 to 23.99 for *P. irregulare* group IV, and 30.95 to 22.50 for *P. ultimum*.

Soil samples were collected from five locations in Whitman County in eastern Washington to test the ability of the real-time PCR procedure to detect *Pythium* spp. directly from natural field soils. Portions of each soil sample were air dried, so that both wet and dry samples were examined. Similar quantities of DNA were amplified from both wet and dry soils, with at least three different species usually amplified from each soil, although only two species of *Pythium* were amplified from the fallow soil collected in Garfield, WA (Table 4). Six species of *Pythium* were detected from the soil cropped to spring barley at Garfield, and *P. irregulare* group IV likely also was present, but quantification was not possible. The quantity of *Pythium* spp. DNA extracted from each soil followed the same trend as with serial dilution and plating soils onto selective media to quantify the population of *Pythium* spp. The lowest amplification with real-time PCR and quantification on selective media was associated with fallow soil at Garfield. The Cunningham Agronomy Farm samples and soil from the ARS Palouse Conservation Farm were intermediate in population and PCR amplification, and the spring barley cropped soil from Garfield was the highest, with the exception of the moist soil from the ARS Palouse Conservation Farm.

Soil extracts were spiked with known amounts of *Pythium* spp. DNA, using a range of 0.01 to 100 pg. Similar efficiencies were calculated for DNA amplification from extracts of all three soils (Table 5). Although slight variations in efficiency were observed, the means did not significantly differ from each other or the water control using Fisher's LSD at $P = 0.05$.

Confirmation of species identified in natural field soils. Sixty-four isolates of *Pythium* spp. were isolated from soil samples by baiting using fresh grass blades, with the majority of the isolates being recovered from soils that were cropped in 2004 versus fallowed (Table 6). *P. rostratifingens* was the most commonly recovered species, representing 48% of the isolates. *P. irregulare* group IV represented 19% of the isolates and *P. abappressorium* represented 13%. Four isolates of *P. heterothallicum* and one isolate of *P. irregulare* were recovered as well as five isolates of *P. pachycaule* and three unidentified isolates. However, two species of *Pythium* amplified by real-time PCR

TABLE 2. Species-specific primers for pathogenic *Pythium* spp.

Species	Primer	Sequence (5'→3')	Product size (bp)	Melting peak (°C) ^a
<i>Pythium abappressorium</i>	ABA1bF ABA1R	GTTGTTGTGCGTCTGCGGATTG TGCATAAACGAATATACCAACCGC	397	84.8
<i>P. attrantheridium</i>	ATT3F ATT2R	GTTTGTGGTCATTTTGGCTGCG CGCTACTAACAAAGCAGATCCCAG	277	88.3
<i>P. heterothallicum</i>	HET4F HET2R	GTGAAGTGTCTCGCGCACTTG GTAACCATGCATGCTGCACCA	304	86.6
<i>P. irregulare</i> group I	IRR3cF IRR3R	GCTGTGGTTGGTGTGTTGTTTGC CTGTACAATTGCACACACACAAGTATG	344	88.9
<i>P. irregulare</i> group IV	IIV7F ABA1R	GTATCGTCTTGGCGGAGTGG TGCATAAACGAATATACCAACCGC	370	85.5
<i>P. paroecandrum</i>	PAR2F PAR3R	TGGTTGGCGTTCGTTGTTTG GGATCAACCCGGAGTACACTAATT	398	89.1
<i>P. rostratifingens</i>	ROS4F ROS3R	GGTGTAGTCCGGCTTGGAGAAGGA GCGCACCGACAAACGCATACC	353	87.0
<i>P. sylvaticum</i>	SYL1F SYL2R	GTGTCTCGCTGTGGTTGGTATATTTG CTTCTGCCAATTGCACAAGTGC	341	88.7
<i>P. ultimum</i>	ULT1F ULT4R	GACACTGGAACGGGAGTCAGC AAAGGACTCGACAGATTCTCGATC	414	84.8

^a Melting peaks were determined by melting the polymerase chain reaction products using the LightCycler (Roche Applied Science, Indianapolis, IN) and continuously recording the fluorescence during the melting process.

were not isolated from soil, including *P. attrantheridium* and *P. ultimum*.

Similar results were obtained when soil extracts from the same locations described above were tested using a DNA macroarray. Five *Pythium* spp. were detected in the Cunningham Agronomy Farm sample and four species each in the Garfield and ARS Palouse Conservation Farm samples. *P. heterothallicum* and *P. rostratifingens* were detected at all three locations, whereas *P. ultimum* was found only in the Garfield sample. Hybridization signals specific for *P. attrantheridium* were detected in the Cunningham Agronomy Farm and ARS Palouse Conservation Farm samples. An oligonucleotide (par131), designed for part of the *paroeacandrum/irregulare* complex, exhibited positive hybridization signals in all samples. However, because the oligonucleotide (par126) specific for *P. paroeacandrum* did not exhibit any hybridization signal, it is highly probable that this species is not present. A *P. torulosum* oligonucleotide also weakly hybridized to the Cunningham Agronomy Farm sample.

DISCUSSION

Identification of *Pythium* spp. by classical methods of morphological features is time consuming and difficult. In this study, primers specific to nine pathogenic *Pythium* spp. commonly found in Washington were developed. These primers subsequently were used to develop an assay to identify and quantify species of *Pythium* from artificially and naturally infested soils, using a soil DNA extraction kit and real-time PCR.

Specific and sensitive detection using real-time PCR was achieved with all nine species of *Pythium* examined. The primer sets that were developed provided specific detection of the species for which they were designed and did not amplify nontarget DNA

Fig. 2. Variability in quantification of DNA extracted from mycelia of cultured *Pythium* spp. using real-time polymerase chain reaction over a range of DNA concentrations (10 ng to 10 fg). Each point represents the standard deviation of three subsamples.

Fig. 1. Standard curves generated using real-time polymerase chain reaction for each *Pythium* species-specific primer set developed for identification and quantification. A range of DNA concentrations from 10 ng to 10 fg was used in generating the above graphs. Each DNA concentration was tested in triplicate. **A**, *P. abappressorium* primer set ABA1bF/ABA1R, **B**, *P. attrantheridium* primer set ATT3F/ATT2R, **C**, *P. heterothallicum* primer set HET4F/HET2R, **D**, *P. irregulare* group I primer set IRR3F/IRR3R, **E**, *P. irregulare* group IV primer set IIV2F/ABA1R, **F**, *P. paroeacandrum* primer set PAR2F/PAR3R, **G**, *P. rostratifingens* primer set ROS4F/ROS3R, **H**, *P. sylvaticum* primer set SYL1F/SYL1R, and **I**, *P. ultimum* primer set ULT1F/ULT4R. The efficiency of each primer set was calculated using the formula: $E = 10^{(-1/\text{slope})} - 1$.

of other *Pythium* spp. or other genera tested in this study. A primer set was designed previously for specific detection of *P. ultimum* and, when used in combination with traditional PCR, could detect *P. ultimum* from water-soaked lesions of cucumber, sugar beet, and Chinese cabbage (22). Wang et al. (50) recently developed a set of 20 PCR primers based on ITS1 sequence from 34 *Pythium* spp. However, they obtained specific detection only for *P. acanthicum*, *P. rostratum*, *P. splendens*, and *P. sulcatum*, with some primers amplifying more than one species. They developed a specific forward primer and used a universal primer (ITS2 or ITS4) as the reverse primer (50). In our study, both the forward and reverse primers were designed to be species specific, with a forward primer in either the ITS1 or ITS2 and a reverse primer in the ITS2. The use of two specific primers greatly increases the specificity of each primer set.

In developing standard curves for each primer set, detection was achieved at a fairly low template concentration of DNA (10 fg). In the development of an assay to detect *Phytophthora capsici* in pepper tissue, as little as 10 pg of target DNA could be detected using SYBR Green in conjunction with a Bio-Rad iCycler (44). In the detection of *Pyrenophora teres*, a standard curve was generated using a range of concentrations from 2.5 ng to 2.5 pg (6). However, using a real-time nested PCR technique, *Phytophthora ramorum* could be detected at concentrations as low as 12 fg (19). Quantification of DNA down to the low femto-gram range also was reported for *Colletotrichum coccodes* when real-time PCR was performed using fluorescently labeled probes in combination with species-specific primers and an ABI Prism 7700 sequence detector (15). Based on amplification of samples from artificially infested soils and directly from the field soils, lower detection limits are likely possible. In testing field samples, as low as 2.6 and 4.5 fg of *P. ultimum* DNA per reaction were detected from soils removed from a spring barley field in Garfield, and 1.4 fg of *P. rostratifingens* DNA per reaction was detected from the Garfield fallow soil.

The standard curves were generated using a range of DNA concentrations from 10 ng to 10 fg. At most DNA concentrations, the real-time PCR amplification was very reproducible, with average standard deviations of 0.033 for the concentration estimates. However, when only 10 fg of DNA were initially present in a reaction, the average standard deviation increased fourfold. Although quantification is still possible using a starting target DNA concentration of only 10 fg, the error increases at these low concentrations. Such increase in standard deviation is expected when the estimates are close to the limit of the range of the standards.

P. irregulare group I, *P. irregulare* group IV, and *P. ultimum* were chosen for amplification directly from soil because these three species previously were demonstrated to be the most virulent on wheat (20,21). The isolates described as *P. irregulare* group IV in this article previously were designated *P. debaryanum* based on morphology and 97% sequence similarity (20,42). How-

ever, these same isolates share 100% sequence identity to isolates that were recovered from sugar beet in Japan and designated *P. irregulare* group IV (31). ITS sequence data evaluated by Lévesque and De Cock (25) also showed that *P. irregulare* group IV isolates occur in the same clade as the more common *P. ir-*

Fig. 3. Correlation of DNA recovered from soil samples with populations of *Pythium* spp. from the same soil. Pasteurized soils were artificially inoculated with **A**, *P. irregulare* group I isolates 110305 (Δ), 110306 ($\circ$), and 0900101 ($\square$); **B**, *P. irregulare* group IV isolates 020117 (Δ), 020147 ($\circ$), and 020155 ($\square$); or **C**, *P. ultimum* isolates 030141 (Δ), 10118 ($\circ$), and 10145 ($\square$). DNA was extracted from the soil using a MO BIO Laboratories soil DNA extraction kit and amplified by real-time polymerase chain reaction using a Roche LightCycler; ppg = propagules per gram.

TABLE 3. Summary of primer specificity using a collection of 77 isolates, representing nine *Pythium* spp.^a

Species	Isolates	Isolates amplified	C _t range (positive)	Nonspecific amplification ^b	C _t range (negative)
<i>Pythium abappressorium</i>	11	11	12.8–16.6	0	35.1–43.7
<i>P. attrantheridium</i>	5	5	13.0–14.8	0	32.0–>46.0
<i>P. heterothallicum</i>	13	13	13.0–15.9	0	29.5–36.7
<i>P. irregulare</i> group I	8	8	13.1–14.2	0	31.3–34.3
<i>P. irregulare</i> group IV	11	11	13.1–15.0	0	29.0–37.3
<i>P. paroecandrum</i>	3	3	13.8–15.3	0	35.4–>46.0
<i>P. rostratifingens</i>	11	11	12.1–14.7	0	32.7–>46.0
<i>P. sylvaticum</i>	2	2	13.1–13.6	0	32.4–>46.0
<i>P. ultimum</i>	13	13	11.5–16.1	0	31.0–43.1

^a All tests for primer specificity were conducted using a Roche LightCycler with SYBR Green I fluorescent dye for detection of polymerase chain reaction product. Positive amplifications were confirmed by examination of melting curves. For each sample, approximately 2 to 3 ng was used per reaction; C_t = cycle threshold.

^b Each primer set was tested with a collection of 77 isolates. Nonspecific amplification implies that a primer amplified an isolate other than the species for which the primer was designed.

regulare, but form a distinct cluster. The taxonomic status of the closely related cluster made by a CBS strain of *P. debaryanum* and some isolates of *P. violae* is unknown at this point. In a separate comparison of 68 isolates of *P. irregulare* based on ITS sequence, *P. irregulare* group IV was found to be more closely related to *P. sylvaticum* than to *P. irregulare* (18), which is consistent with the analysis of Lévesque and De Cock (25). *P. irregulare* group IV isolates in this study have numerous aplerotic oospores, with one or two monoclinal antheridia arising very close to the oogonium.

A method of DNA extraction was modified from a kit and real-time PCR amplification from soils was achieved. Using pasteurized soils that were artificially infested with *Pythium* spp., this technique was used to detect as few as 10 ppg. Similar sensitivity would be expected for the other primer sets developed in this study. In addition, greater sensitivity than observed under controlled conditions in this study is likely. In developing TaqMan-based real-time PCR assay to detect *Phytophthora medicaginis* in alfalfa roots, resistant versus susceptible plants could be differentiated with an average of 50 pg of DNA detected in resistant plants (47). Bates et al. (6) detected <2% seed infection with *Pyrenophora* spp. using a similar assay that involved real-time PCR by means of a LightCycler and SYBR Green dye. Likewise, detection of as few as 1.5 conidia of *Helminthosporium solani* per gram of soil was demonstrated using TaqMan fluorogenic probes in combination with a 96-well format quantitative PCR (14).

The soil DNA extractions were highly reproducible; however, slight variability did exist between isolates. One possibility that was examined was that the copy number of nuclear rDNA differed between isolates. However, a simple test was conducted in which DNA from several isolates of *P. ultimum* was standardized to the same concentration. Hence, the number of genomes per 1 µl of DNA should be approximately the same. A 10-fold dilution series was constructed and each dilution was amplified using real-time PCR. The result was virtually identical C_t values at all dilutions for each isolate, indicating very similar copy numbers per isolate.

The second and more likely possibility was that the population estimates made by dilution plating onto selective media were less precise than quantification of DNA. Previous research has demonstrated variability between PCR results and plating of samples onto selective medium. Kageyama et al. (22) found that *P. ultimum* could be detected in infected plant samples by PCR amplification, yet was undetectable when the same samples were placed onto a selective medium. Thus, use of selective media to quantify *Pythium* spp. directly from soil or any other substrate is less sensitive than PCR techniques and is only a rough estimate. Finally, *Pythium* spp. possess a number of different types of propagules, including sporangia, hyphae, and oospores. Sporangia can germinate readily and grow; whereas, with some species of *Pythium*, the oospores must undergo a period of dormancy before being capable of germinating (3,28).

The population dynamics of *Pythium* spp. in soil is complex. In each of the soil samples examined in this study, between two and six pathogenic species were found per soil sample. Similarly, in a

TABLE 5. Percent amplification efficiency for soil extracts spiked with known amount of DNA^a

Soil ^b	Efficiency (%) ^c		
	<i>Pythium irregulare</i> group I	<i>P. irregulare</i> group IV	<i>P. ultimum</i>
Cunningham	89.0	89.1	83.6
Garfield	91.7	91.9	85.3
ARS-PCFS	88.7	88.8	84.8
Control	88.9	88.8	83.4

^a Soil extracts were spiked with 100 pg of *Pythium* spp. DNA and serial dilutions were made down to 10 fg. Values are the average of three replications.

^b Soils were collected from the Cunningham Farm (Cunningham), Garfield, and the Agricultural Research Service Palouse Conservation Farm (ARS-PCFS). Control = water only.

^c Efficiency was calculated using the formula: $E = 10^{(1-\text{slope})} - 1$. None of the differences in amplification efficiency were statistically significant using Fisher's least significant difference ($P = 0.05$).

TABLE 4. Summary of amplification of indigenous *Pythium* spp. from natural field soils using species-specific primers and real-time polymerase chain reaction (PCR)

Species	DNA quantification from field soils (fg/g of soil) ^a				
	Cunningham WW	Cunningham fallow	Garfield SB	Garfield fallow	ARS-PCFS SW
Wet soils					
<i>Pythium abappressorium</i>	2,735	6,065	680	—	—
<i>P. attrantheridium</i>	—	—	1,595	—	1,025
<i>P. heterothallicum</i>	6,000	7,290	1,565	—	(440)
<i>P. irregulare</i> group I	—	—	7,980	—	—
<i>P. irregulare</i> group IV ^b	—	?	?	—	?
<i>P. paroecandrum</i>	—	—	—	—	—
<i>P. rostratiformis</i>	5,810	4,505	7,960	—	1,730
<i>P. sylvaticum</i>	—	—	—	—	—
<i>P. ultimum</i>	—	—	(130)	—	—
Total (ppg) ^c	1,210	1,130	1,300	570	2,070
Dry soils					
<i>P. abappressorium</i>	2,775	2,505	580	(490)	—
<i>P. attrantheridium</i>	—	—	4,445	—	3,045
<i>P. heterothallicum</i>	3,705	4,730	3,740	—	550
<i>P. irregulare</i> group I	—	—	5,360	—	—
<i>P. irregulare</i> group IV ^b	—	—	?	—	—
<i>P. paroecandrum</i>	—	—	—	—	—
<i>P. rostratiformis</i>	7,945	2,895	1,890	(70)	2,870
<i>P. sylvaticum</i>	—	—	—	—	—
<i>P. ultimum</i>	—	—	(225)	—	—
Total (ppg) ^c	410	650	1,280	200	960

^a DNA was quantified from each sample real-time PCR using a Roche LightCycler and SYBR Green I fluorescent dye for detection of the PCR product. In samples with a dash (—), *Pythium* was not detected. Sample with parentheses were detected and quantified, but amplification was <10 fg per reaction. Soils were collected from the Cunningham Farm (Cunningham), Garfield, and the Agricultural Research Service Palouse Conservation Farm (ARS-PCFS). WW = winter wheat, SB = spring barley, and SW = spring wheat.

^b Amplification of *P. irregulare* group IV was detected in several of the soil samples (indicated by a question mark), but could not be accurately quantified due to a background product that was formed with the IIV2F/ABA1R primers when target DNA concentrations were low.

^c *Pythium* spp. total population in propagules per gram (ppg).

recent survey of *Pythium* spp. from eastern Washington, up to six species were recovered from a single soil sample (42). Quantification and identification also followed closely with the previous history at each location. The highest recovery in both plate counts on selective media and from quantitative PCR was from the spring barley field in Garfield. Lower populations were detected in the cropped soils at the Cunningham Agronomy Farm and the ARS Palouse Conservation Farm. The lowest population was from the second field at Garfield that was maintained in mechanical summer fallow over the 2004 growing season. Populations recovered from the fallow soil at the Cunningham Agronomy Farm were very similar to those from the cropped soil. The fallow soil was a patch of land that was being maintained by chemical fallow within a wheat field. However, later in the growing season, these patches became overgrown in weeds. In addition, the field was harvested prior to sampling from the fallow area and sampling was preceded by several days of rainfall. In controlled growth-chamber studies, Cook et al. (12) demonstrated that the addition of wheat chaff to a soil could substantially increase the population of *Pythium* spp. within 4 days. Thus, the combination of wheat chaff spread over the fallow areas and rain after harvest may have contributed to similar populations between fallow and cropped soils at the Cunningham Agronomy Farm.

The morphological identification of *Pythium* spp. from field soils examined in this study revealed similar information about population dynamics when compared with the real-time PCR data. *P. rostratifingens*, *P. irregulare* group IV, and *P. abappressorium* represented the most commonly isolated species based on morphological data and higher quantities of DNA were amplified for these same species using real-time PCR, although specific quantities of DNA could not be determined for *P. irregulare* group IV. The primer set that was used for *P. irregulare* group IV was an earlier version that performed unacceptably in soils, resulting in high nontarget background amplification. A sample was not available to be retested with the improved IIV7F/ABA1R primer set. Likewise, *P. paroecandrum* and *P. sylvaticum* were not recovered from grass blades and were not observed using real-time PCR. However, several species that were detected by real-time PCR were not isolated from the soil. In the Garfield soil cropped to spring barley, similar levels of amplification with real-time PCR were detected for *P. attrantheridium*, *P. heterothallicum*, *P. irregulare*, and *P. rostratifingens*; however, only *P. irregulare* and *P. rostratifingens* were recovered from soil by baiting. Many more isolates likely would need to be examined to recover all of the species that were identified by real-time PCR, and the proportion of species recovered likely would differ from that observed with

real-time PCR due to experimental variability in the recovery of each species by baiting.

Similar results also were observed using the DNA macroarray. As with the results from real-time PCR, *P. heterothallicum* and *P. rostratifingens* were detected from all three field locations, *P. ultimum* was found only in Garfield soil, and *P. paroecandrum* and *P. sylvaticum* were not present in any of the samples. Similar results also were observed for *P. attrantheridium*, although the DNA macroarray detected this species at the ARS Palouse Conservation Farm but not at Garfield, contrary to the results of real-time PCR. *P. abappressorium* and *P. irregulare* group IV were not detected by DNA macroarray. However, BLAST analysis of the *paroecandrum* group oligonucleotide (par131) against 500 *Pythium* entries in GenBank revealed a perfect match to sequences of *P. abappressorium*, a species with no specific oligonucleotide on the array. *P. irregulare* group IV also does not yet have a specific oligonucleotide on the array; therefore, no supportive data is available for this species.

A useful feature of real-time PCR as performed using the Roche LightCycler is the ability to examine the melting curves of the products formed to check the purity and confirm the identity of the product. Each product formed by PCR amplification will have a distinct melting curve based on the length and base composition of the product. In field samples, a very small amount of background amplification was present in negative samples using several of the primer sets. However, by examining the melting curve and peaks, positive samples easily can be distinguished from nontarget amplification. In the case of *P. irregulare* group IV, specific amplification occurred in three of the soils. However, some of the amplification was due to nontarget amplification. By examining the melting profile, the presence or absence of *P. irregulare* group IV could still be discerned in the three soils.

Despite examining only a few field samples, similarities to previous work were evident. Paulitz and Adams (42) found six species of *Pythium* to occur at frequencies above 25%. In order from highest recovery to lowest, they were *P. abappressorium*, *P. rostratifingens* (aff. *P. rostratum*), *P. irregulare* group IV (aff. *P. debaryanum*), *P. heterothallicum*, and *P. ultimum*. The *P. rostratum* referred to by Paulitz and Adams (42) recently was classified as *P. rostratifingens* based on ITS sequence and various morphological factors, including the absence of catenulate oogonia and differences in oogonia and sporangia size (16). In this study, the highest frequency of specific amplification using real-time PCR was with *P. heterothallicum* and *P. rostratifingens* primers sets, with both species being identified from four of the five soils. *P. abappressorium* and *P. irregulare* group IV also were observed

TABLE 6. *Pythium* spp. isolated from field soils that were identified by morphological features and confirmed by sequencing of representative isolates and compared to a database (25)

Species	Number of isolates per field soil sample ^a				
	Cunningham WW	Cunningham fallow	Garfield SB	Garfield fallow	ARS-PCFS SW
<i>Pythium abappressorium</i>	5	—	—	1	2
<i>P. attrantheridium</i>	—	—	—	—	—
<i>P. heterothallicum</i>	—	3	—	1	—
<i>P. irregulare</i> group I	—	—	1	—	—
<i>P. irregulare</i> group IV	1	3	4	—	4
<i>P. paroecandrum</i>	—	—	—	—	—
<i>P. pachycaule</i> ^b	2	—	2	1	—
<i>P. rostratifingens</i>	11	—	6	—	14
<i>P. sylvaticum</i>	—	—	—	—	—
<i>P. ultimum</i>	—	—	—	—	—
Unidentified	—	—	2	1	—
Total	19	6	15	4	20

^a Isolates were baited from soils using fresh grass blades. After obtaining pure cultures, identifications were made by incubating agar plugs containing the *Pythium* spp. in pond water with sterilized, dried grass blades and examining the sexual and asexual features of each isolate. Samples with a dash (—) indicate the particular species was not among the isolates examined. Soils were collected from the Cunningham Farm (Cunningham), Garfield, and the Agricultural Research Service Palouse Conservation Farm (ARS-PCFS). WW = winter wheat, SB = spring barley, and SW = spring wheat.

^b *P. pachycaule* Ali-Shtayeh has filamentous sporangia and is closely related to *P. dissotomicum* Drechsler. This species was identified by sequencing only.

at a high frequency in three of five samples. *P. irregulare* and *P. ultimum* were identified from one of five samples, which also closely follows the observations of Paulitz and Adams (42). Thus, the real-time PCR technique appears to produce results similar to those obtained with a large-scale survey.

Traditional methods of enumerating and identifying *Pythium* spp. rely on the use of selective media and microscopic identification of reproductive features of each isolate. These processes are laborious, requiring up to 8 to 14 days for species identification. This same process can be accomplished in only a few hours using soil DNA extraction techniques and real-time PCR. In addition, real-time PCR is a very sensitive technique, capable of detecting low population densities of certain *Pythium* spp. that might be overlooked by traditional methods of screening.

In this study, primers were developed for the nine most commonly isolated pathogenic *Pythium* spp. in Washington. However, over 120 species of *Pythium* have been described (2). Because a comprehensive collection of *Pythium* spp. was not screened, some nonspecific amplification might occur with species of *Pythium* not commonly found in eastern Washington. Both primers for each set, with the exception of the reverse primer for *P. abappressorium* and *P. irregulare* group IV, were designed to specifically amplify only one species. Because both primers were designed to be specific, the likelihood of both primers binding to sequences of nontarget species is reduced. This is supported by findings from the in silico tests. In the pairings where ΔG values were low, only one primer of the pair was predicted to form a hybrid. Therefore, the potential for double-stranded, nonspecific amplification was considered to be nil. The exception was the pairing of PAR3R and SYL1F, which was predicted to amplify *P. sylvaticum* ITS DNA. Thus, there would be a limitation in adopting these primer sets for multiplexing in future assays. Further testing of these primers with local species also may be necessary if this procedure were adopted in other areas.

The method for identification and quantification outlined in this article will serve as a useful tool for study of *Pythium* spp. The technique is very sensitive and fast compared with traditional methods of dilution plating and microscopic observation of morphological features. There are numerous applications for this new technique that will greatly improve our understanding of *Pythium* spp. and the diseases that they cause. The use of this technique will be very beneficial for studying the epidemiology of this pathogen in soils, including how geography, temperature, moisture, and cropping systems affect the species composition of this pathogen. It also will prove useful from a disease management perspective for the evaluation of various control measures, including fungicide sensitivity, quantitative host resistance, and various cultural management strategies.

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