Techniques

Detection and Identification of Four Common Rust Pathogens of Cereals and Grasses Using Real-Time Polymerase Chain Reaction

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ABSTRACT

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Puccinia spp. are widespread pathogens of cereals and grasses that annually cause significant yield losses worldwide, especially in barley, oat, and wheat. Urediniospore morphology and early symptom development have limited usefulness for distinguishing *Puccinia* spp. Therefore, we developed real-time polymerase chain reaction assays for rapid detection of the four rust pathogen species, *Puccinia graminis* (Pers.:Pers.), *P. striiformis* (Westend.), *P. triticina* (Eriks.), and *P. recondita* (Roberge ex Desmaz.). Duplex assays were constructed for the nuclear rDNA gene,

using the variable internal transcribed spacer 1 (ITS1) region to distinguish between species, and the conserved 28S region as an internal control. Species-specific ITS1 primer/probe sets were highly specific and could detect <1 pg of DNA. The species-specific primer/probe sets showed positive results over a linear range of DNA five orders of magnitude or greater. Specificity of the assays was tested using multiple collections representing a range of races and formae speciales within a species. Additionally, assay specificity was evaluated by testing a range of other grass rust pathogens, as well as other fungi. The 28S primer/probe combination was successful in detecting all *Puccinia* spp. tested within the duplex assays, validating the integrity of each assay. Finally, the assays were used to identify unknown rust fungi infecting pasture grasses.

Puccinia spp. are important pathogens of many grasses, but are most notorious for the destructiveness on grain crops, especially wheat. Three *Puccinia* spp. are responsible for the majority of wheat losses: *Puccinia graminis*, *P. striiformis*, and *P. triticina* (37). However, although *P. triticina* is restricted to infecting wheat, *P. graminis* (15,34) and *P. striiformis* (19,30,33) have a relatively broad host range that includes pasture grasses and other wild grass species. Rapid detection and correct identification are important for an increased understanding of the epidemiology of rust diseases of cereal crops and pasture grasses, and for the study of naturally occurring populations of these rust pathogens.

Available methods to detect and verify a given rust fungal species are often imprecise or slow. Rust fungal species are difficult to identify in early stages of disease development, on natural grass hosts, or by urediniospore morphology. Although uredinia of some species become distinctive following a week to 10 days of disease development (*P. graminis*), others are not. Molecular methods, such as conventional polymerase chain reaction (PCR) with Southern hybridization or sequence analysis, can be useful at any stage of development, but are often labor intensive and can require several days to confirm the identity of a sample (11,24, 27). Furthermore, sequence-based methods are limited by low DNA concentrations. Real-time PCR with fluorogenic probes is used to detect and quantify plant pathogens rapidly (8,9,14,26, 38). The advantages of real-time PCR with a fluorogenic probe are that it adds additional specificity beyond the primers, increases sensitivity of PCR through fluorescence detection, and negates the need for post-PCR analysis (6,20,29).

A commonly reported genomic region employed to distinguish fungal species based on sequence differences is nuclear ribosomal

DNA (rDNA) (5,9,12,14,21,28,32,40). The advantages of this region are that it is highly repetitive (24), the rDNA sequences for many fungi are publicly available, and the internal transcribed spacer (ITS) regions evolve relatively quickly (36), making distinction between *Puccinia* spp. possible (40). Confidence in the specificity of a real-time PCR assay depends on knowledge of the variation in the DNA sequence both within the target organism and between the target and closely related nontarget organisms.

The objective of this study was to develop a real-time PCR system to rapidly detect and identify rust fungal species when conventional identification by disease symptom or spore morphology is not possible, or in instances when only a limited number of spores are available, such as from rain or air collectors. The concept in the assay design was to employ species-specific fluorogenic probes and general rust-fungus-specific primers and PCR conditions. In this study, we developed species-specific real-time PCR assays for the independent detection of four common rust fungi that infect cereals and grasses, *P. graminis*, *P. striiformis*, and *P. triticina;* and *P. recondita* f. sp. *secalis*, which infects rye (*Secale cereale* L.) and wild relatives of wheat (*Aegilops* spp.). Each assay consisted of (i) rust-fungus-specific primers for the ITS1 region and *Puccinia* spp.-specific TaqMan 6-carboxy-fluorescein (FAM) probes and (ii) general primers for the 28S region and a nonspecies-specific TaqMan TexasRed probe as an internal positive control.

METHODS

***Puccinia* spp. used for assay development.** *Puccinia* spp. samples were used for two purposes in this study: (i) to obtain DNA sequences for interspecific and intraspecific alignments for primer and probe design and (ii) to validate the specificity of the real-time PCR assays. Fungal samples used in this study are shown in Table 1. *Puccinia* spp. samples for both purposes were chosen to be representative of a broad population of each rust fungal species and included samples that varied by where and when they were collected, by host, and by race, when applicable.

DNA extraction. DNA was extracted from either collected, dried urediniospores (1 to 20 mg) or from diced, infected leaf tissue with visible uredinia (20 to 30 mg). All samples were shaken in 2-ml tubes with 1 mm of glass beads (Lysing Matrix C; Bio 101, Carlsbad, CA) and 25 mg of diatomaceous earth (Sigma-Aldrich, St. Louis) in a Savant FastPrep shaker (FP120; Holbrook, NY) for 10 s at a speed setting of 5 (2). DNA was extracted using the following modifications of the cetyltrimethylammonium bromide (CTAB) method described by Liu and Kolmer (16) and Anikster et al. (2). Following incubation at 65°C for 1.5 h in 600 µl of extraction buffer (0.165 M Tris-HCl, pH 8.0; 66 mM EDTA, pH 8.0; 1.54 M NaCl; 1.1% CTAB; and Proteinase K at 50 µg/ml) and 66 µl of 20% sodium dodecyl sulfate, samples were extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, vol/vol/vol), followed by extraction with an equal volume of chloroform:isoamyl alcohol (24:1, vol/vol). The aque-

TABLE 1. List of fungal samples used in this study

Species	Race	Host	Origin	Source[a]	Year	GenBank accession no.
Rust fungi						
Puccinia graminis						
57KS17-2C	MCCF	*Triticum aestivum*	Kansas	1	1957	DQ417374
61PA80A	RFQQ	*T. aestivum*	Pennsylvania	1	1961	DQ417373
80MN518-3	RKRQ	*T. aestivum*	Minnesota	1	1980	DQ417376
59OH5B	QCCQ	*T. aestivum*	Ohio	1	1959	DQ417375
70MEX3A	RTHJ	*T. aestivum*	Mexico	1	1970	DQ417377
89OR436B	QFCS	*T. aestivum*	Oregon	1	1989	DQ417380
68MO192-1A	RHTS	*T. aestivum*	Missouri	1	1968	DQ417379
61IA21C	TPMK	*T. aestivum*	Iowa	1	1961	DQ417378
HSZ0009	...	*Elymus trachycalus*	Wisconsin	1	2001	DQ417381
HSZ0931	...	*T. aestivum*	Switzerland	1	2003	DQ417387
HSZ0679	...	*T. aestivum*	Czech Republic	2	2002	DQ417382
HSZ0920	...	*Phleum pratense*	Minnesota	1	2001	DQ417392
HSZ0922	...	*Elytrigia repens*	Switzerland	1	2003	DQ417388
HSZ0929	...	*Poa pratensis*	Switzerland	1	2003	DQ417389
HSZ0928	...	*Dactylis glomerata*	Switzerland	1	2003	DQ417390
HSZ0926	...	*Avena sativa*	Switzerland	1	2003	DQ417391
78-21-BB463[b]	DFBJ	*T. aestivum*	Pennsylvania	1	1978	AY114289[c]
56SD37B[b]	RCRS	*T. aestivum*	South Dakota	1	1956	DQ417383
HSZ0802[b]	...	*Lolium perenne*	Oregon	1	2003	DQ417384
HSZ0803[b]	...	*Festuca arundinacea*	Oregon	1	2003	DQ417385
HSZ0753[b]	...	*Anthroxanthum* spp.	Czech Republic	2	2003	DQ417386
84FL680[b]	RCRS	*T. aestivum*	Florida	1	1984	...
74KS264A[b]	TPMK	*T. aestivum*	Kansas	1	1974	...
80MN633B[b]	TPMK	*T. aestivum*	Minnesota	1	1980	...
75WA205A[b]	RHTS	*T. aestivum*	Washington	1	1975	...
91MEX647[b]	RTRQ	*T. aestivum*	Mexico	1	1991	...
72CAN1321A[b]	MCCD	*T. aestivum*	Canada	1	1972	...
73VA441A[b]	MCCD	*T. aestivum*	Virginia	1	1973	...
93NY224B[b]	QFCS	*T. aestivum*	New York	1	1993	...
91KS60A[b]	QFCS	*T. aestivum*	Kansas	1	1991	...
91WA496A[b]	QFCS	*T. aestivum*	Washington	1	1991	...
P. recondita						
80WV271[b]	...	*Secale cereale*	West Virginia	1	1980	DQ417420
82WI47[b]	...	*S. cereale*	Wisconsin	1	1982	DQ417421
91TX9503[b]	...	*S. cereale*	Texas	1	1991	DQ417422
HSZ0698[b]	...	*S. cereale*	Czech Republic	2	2002	DQ417423
ANK9958[b]	...	*Aegilops ovata*	Israel	3	2002	DQ417424
ANK9974[b]	...	*A. variabilis*	Israel	3	2003	AY187088[c]
HSZ0716	...	*S. cereale*	California	1	2003	DQ417425
HSZ0712	...	*S. cereale*	California	1	2003	DQ417426
ANK77081	...	*T. turgidum* spp. durum	Israel	3	2002	AF511082[c]
P. striiformis						
PSH13	...	*Hordeum vulgare*	Washington	4	2001	DQ417408
PSH14[b]	...	*H. vulgare*	Washington	4	2001	DQ417393
PST17	...	*T. aestivum*	Washington	4	2001	DQ417394
PST29[b]	...	*T. aestivum*	Washington	4	2001	DQ417395
PST78[b]	...	*T. aestivum*	Washington	4	2001	DQ417396
22/99	...	*T. aestivum*	Denmark	5	1999	DQ417397
HSZ0728	...	*T. aestivum*	Texas	1	2003	DQ417398
HSZ0727	...	*T. aestivum*	Texas	1	2003	DQ417399
HSZ0725	...	*T. aestivum*	Texas	1	2003	DQ417400
HSZ0718	...	*T. aestivum*	Texas	1	2003	DQ417401
HSZ0711	...	*H. vulgare*	California	1	2003	DQ417402
HSZ0699	...	*Dactylis glomerata*	Czech Republic	2	2002	DQ417403

(continued on next page)

[a] Samples from 1, United States Department of Agriculture-Agricultural Research Service, Cereal Disease Laboratory, St. Paul, MN; 2, J. Markova, Charles University, Prague; 3, Y. Anikster, Institute of Cereal Crops Improvement, Tel Aviv, Israel; 4, X. Chen, United States Department of Agriculture–Agricultural Research Service, Wheat Genetics, Quality, Physiology and Disease Research, Pullman, WA; 5, A. F. Justesen, Faculty of Agricultural Sciences, Flakkebjerg, Denmark, DNA; 6, A. del Olmo, CIFA, Córdoba, Spain, DNA; and 7, P. Hamm, Oregon State University, Hermiston.
[b] Rust fungal species used for assay verification.
[c] Previous submissions to GenBank.

ous phase was removed, and nucleic acids were precipitated with 0.6 volume of isopropanol and finally resuspended in 100 μl of Tris-EDTA (TE) buffer (10 mM Tris-HCl, pH 8.0; 0.1 mM EDTA, pH 8.0). RNA was digested by incubation with 3.5 μl of RNase A (10 mg ml⁻¹) at 37°C for 1 h. To precipitate DNA, 0.1 volume of 3.0 M NaOAc and 2.0 volumes of cold 95% ethanol were added to samples, mixed by inversion, and incubated at –20°C for 1 h. After centrifugation, the pellet then was washed twice with cold 70% ethanol, air dried, and resuspended in 100 μl of TE buffer. The

DNA was quantified using a DyNA Quant200 Fluorometer (Hoefer Inc., San Francisco)) and verified by electrophoresis through an agarose gel. DNA from uninfected wheat (*Triticum aestivum*) was provided by David Gavin of the United States Department of Agriculture–Agricultural Research Service, Plant Science Research Unit, St. Paul, MN.

DNA sequencing. rDNA was PCR amplified in a 50-μl reaction mixture as described in Anikster et al. (2). DNA sequencing reactions were performed using a Thermo Sequencase Prime

TABLE 1. (*continued from preceding page*)

Species	Race	Host	Origin	Source[a]	Year	GenBank accession no.
P. striiformis (*continued*)						
HSZ0010	…	*Poa pratensis*	Georgia	1	2001	DQ417407
52/99[b]	…	*T. aestivum*	Denmark	5	1999	DQ417404
HSZ0722[b]	…	*T. aestivum*	Texas	1	2003	DQ417405
HSZ0724[b]	…	*T. aestivum*	Texas	1	2003	DQ417406
PSH31[b]	…	*H. vulgare*	Washington	4	2001	…
P. triticina						
HSZ0741	…	*T. aestivum*	Czech Republic	1	2002	DQ417409
HSZ0742	…	*T. aestivum*	Czech Republic	1	2002	DQ417410
HSZ0743	…	*T. aestivum*	Czech Republic	1	2002	DQ417411
HSZ0744	…	*T. aestivum*	Czech Republic	1	2002	DQ417412
HSZ0745	…	*T. aestivum*	Slovakia	1	2002	DQ417413
HSZ0746	…	*T. aestivum*	Slovakia	1	2002	DQ417414
HSZ0747	…	*T. aestivum*	Slovakia	1	2002	DQ417415
HSZ0748	…	*T. aestivum*	Czech Republic	1	2002	DQ417416
ANK9973	…	*T. turgidum* spp. durum	Israel	3	2002	DQ417417
ANK9538	…	*T. aestivum*	Israel	3	2002	DQ417418
00LA87	…	*T. aestivum*	Louisiana	1	2000	AF511083[c]
98EGY166C[b]	MCDLQS	*T. aestivum*	Egypt	1	1998	AY187087[c]
98EGY151C[b]	PCBCLS	*T. aestivum*	Egypt	1	1998	DQ417419
99NC28-1[b]	TLGF	*T. aestivum*	North Carolina	1	1999	…
93WA504[b]	NBBJ	*T. aestivum*	Washington	1	1993	…
82TX176[b]	FLLQ	*T. aestivum*	Texas	1	1982	…
82MN246[b]	TDBJ	*T. aestivum*	Minnesota	1	1982	…
87NY678[b]	PBMQ	*T. aestivum*	New York	1	1987	…
00CA135[b]	MBGJ	*T. aestivum*	California	1	2000	…
90KS285[b]	MGBD	*T. aestivum*	Kansas	1	1990	…
00EGY38B[b]	MCNLQS	*T. aestivum*	Egypt	1	2000	…
00EGY200C[b]	LBBLBQ	*T. aestivum*	Egypt	1	2000	…
98IL324[b]	MDRQ	*T. aestivum*	Illinois	1	1998	…
98AR243[b]	MDRQ	*T. aestivum*	Arkansas	1	1998	…
99SPN40AO[b]	…	*T. durum*	Spain	6	1999	…
P. andropogonis						
HSZ0261[b]	…	*Andropogon gerardii*	Wisconsin	1	2001	…
HSZ0222[b]	…	*A. gerardii*	Minnesota	1	2002	…
HSZ0237[b]	…	*A. gerardii*	Minnesota	1	2002	…
HSZ0227[b]	…	*Schizachyrium scopari*	Minnesota	1	2002	…
Unknown						
HSZ0882[b]	…	Tall pasture grass	Oregon	7	2003	…
HSZ0883[b]	…	Wide blade grass	Oregon	7	2003	…
HSZ0884[b]	…	Tall pasture grass	Oregon	7	2003	…
HSZ0885[b]	…	Tall pasture grass	Oregon	7	2003	…
HSZ0886[b]	…	*Poa pratensis*	Oregon	7	2003	…
HSZ0887[b]	…	Wide blade grass	Oregon	7	2003	…
P. bromina						
HSZ0663[b]	…	*Bromus inermis*	Czech Republic	2	2002	…
HSZ0664[b]	…	*B. inermis*	Czech Republic	2	2002	…
P. arrhenathericola						
HSZ0659[b]	…	*Arrhenatherum elatius*	Slovakia	2	2002	…
P. triseti						
HSZ0700[b]	…	*Trisetum flavescens*	Czech Republic	2	2002	…
P. coronata						
HSZ0425[b]	…	*Rhammus catharticus*	Slovakia	2	2002	…
P. persistens						
HSZ0688[b]	…	*Elytrigia intermedia*	Czech Republic	2	2002	…
Tranzschelia fusca[b]	…	*Anemone quinquefolia*	Minnesota	1	2003	…
T. pruni-spinosae[b]	…	*A. quinquefolia*	Minnesota	1	2003	…
Other fungi						
Ustilago maydis	…	*Zea mays*	Minnesota	1	1998	…
Fusarium graminearum	…	*Triticum aestivum*	Minnesota	1	2003	…

Cycle sequencing kit (Amersham Biosciences, Piscataway, NJ) and analyzed on an automated DNA sequencer (LI-COR, Lincoln, NE). Three or more clones were sequenced for each sample, and the DNA sequence data was assembled and edited with Sequencer (Genecodes, Ann Arbor, MI). Nucleotide sequence data have been submitted to GenBank, with the accession numbers listed in Table 1.

Primer and probe design. Primer and probe sequences are shown in Table 2. Sequence alignments of the rDNA region of *Puccinia* spp. were used to design two pairs of oligonucleotide primers. The first primer pair, ITS1rustF10d and ITS1rustR3c, amplify the ITS1 region, producing rust-fungus-specific fragments ranging from 260 to 270 bp among the four *Puccinia* spp. (data not shown). The second primer pair, StdLSUF5a and StdLSUR2a, was used as an internal standard control and amplified the highly conserved 28S large subunit (LSU) region. To distinguish among the four *Puccinia* spp., species-specific TaqMan probes were designed in the ITS1 region, each labeled at the 5′ end with the reporter dye FAM and the fluorescent quencher dye 6-carboxy-tetramethylrhodamine (TAMRA) at the 3′ end (IDT, Coralville, IA). A probe to the conserved 28S region sequence was designed as an internal standard for fungal rDNA. The ITS1 primers and probe and the StdLSU primers and probe were combined in a single-tube duplex assay for each species. The internal standard TaqMan fluorogenic probe was labeled at the 5′ end with the reporter dye TexasRed-X (succinimidyl ester) (Invitrogen, Carlsbad, CA,) and at the 3′ end with the fluorescent quencher dye BHQ-2 (poly-azo poly-aromatic compound) (Biosearch Technologies, Inc., Novato, CA). All primer pairs and the corresponding probes were designed by comparing sequence alignments for *Puccinia* spp. listed in Table 1 using MacVector (version 7.2; MacVector Inc., Cary, NC).

Real-time PCR conditions and analysis. Reactions were performed using a SmartCycler (Cepheid, Sunnyvale, CA) in 25-μl Cepheid tubes. Optimal conditions were determined by performing the real-time assays with concentration gradients of primers (300, 600, and 1,000 nM) and MgCl₂ (0.5 to 4.0 mM) at different annealing temperatures (58 to 64°C). The threshold cycle (C_T) value was recorded for each dye as the cycle at which the fluorescent signal, associated with an exponential amplification of the PCR product, intersects the threshold set at 30. Conditions were considered optimal if they produced the lowest C_T values and the highest fluorescence over all four *Puccinia* assays. The final reaction conditions used in this study consisted of 25-μl volumes containing 2 μl of sample DNA (0.5 to 1.0 ng μl⁻¹) and 23 μl of a master mix containing 1.0 μM each of the four primers, 0.2 μM both TaqMan fluorogenic probes, 0.2 μM dNTPs, 1× PCR buffer, 2 mM MgCl₂, 0.625 units of Taq polymerase (Applied Biosystems, Branchburg, NJ), and 0.005% casein. Amplification of all samples was based on the following conditions: initial denaturation at 95°C for 2 min, followed by 45 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Assay results were represented graphically by exporting data points from the SmartCycler software and importing them into DeltaGraph 5.6 (Red Rock Software, Salt Lake City, UT).

Assay sensitivity and specificity. Sensitivity limits were determined using genomic DNA starting at 1 to 10 ng and serially diluted 10-fold until the target DNA could not be detected. Standard curves for each *Puccinia* spp. were generated by plotting the log of the known DNA concentration on the *y* axis against the C_T value generated by the second derivative (2nd derivative-C_T) on the *x* axis. The second derivative is the measure of the rate of increase of the amplicon, measured as the increase in fluorescence. The 2nd derivative-C_T is the cycle at which the greatest increase in fluorescence occurred. The simple linear regression line and coefficient of determination (r^2) were calculated by the SmartCycler software within a single experiment. Real-time PCR reaction efficiency was measured by the slope of the line of the log of sample dilutions of known DNA concentrations plotted against the 2nd derivative-C_T values as E = $(10^{(-slope)} - 1) * 100$, with the 2nd derivative-C_T value on the *x* axis. In this study, reaction efficiency was monitored mainly as a measure of PCR inhibition in any reaction when a series of DNA concentrations were known.

FAM probe specificity was considered true only if there was a positive C_T for DNA from all isolates of a given species and a zero C_T for all other DNA tested. In all, 1 ng to ≈500 pg of DNA for each sample was used to determine assay specificity. Amplification for both primer pairs was confirmed by agarose gel electrophoresis.

Assay application. In the fall of 2004, six samples of rust fungi infecting five unknown pasture grass hosts received preliminary identification in the field and by light microscopy. To confirm the species identity of the rust fungus from each sample, real-time PCR assays were performed and PCR amplicons were cloned and sequenced.

RESULTS

Primer and probe design. In order to increase our confidence in the specificity of the primers and probes, the ITS region of a wide collection of samples was sequenced (Table 1). A segment of ≈1,300 bp containing the 5′ end of the 18S complete ITS region and 5′ end of the 28S rDNA was sequenced for 20 samples of *P. graminis*, 7 samples of *P. recondita* f. sp. *secalis*, 16 samples of *P. striiformis*, and 11 samples of *P. triticina*. The sequence alignment showed ITS1 to have a higher level of variation than ITS2; therefore, this region was used for primer and probe design.

The primer pair of the regions flanking the ITS1 (ITS1rustF10d and ITS1rustR3c) amplified all the rust fungal species tested (Table 3), but did not amplify *Ustilago maydis*, *Fusarium graminearum*, or *T. aestivum* (Fig. 1A). Design of species-specific FAM probes was based on selective sites identified to maximize

TABLE 2. Polymerase chain reaction primer and fluorescent probe sequences used in this study

Primer/probe	Sequence (5′–3′)	Tm (ºC)[a]	Location (bp)[b]
ITS1rustF10d	TGAACCTGCAGAAGGATCATTA	51.6	47–68
ITS1rustR3c	TGAGAGCCTAGAGATCCATTGTTA	51.9	293–316
StdLSUF5a	CGTGAGGGAAAGATGAAAAGAA	52.8	1,009–1,030
StdLSUR2a	AGACTGCTACATTTCCTTAAACCC	52.1	1,173–1,196
Pg FAM 1[c]	FAM-TTGTGGCTCGACTCTCTTATAAACCAAACC-TAMRA	68.6	93–122
Pr FAM 2[c]	FAM-TTACCCAACTTTACCAAGACTTCTTTGCATGA-TAMRA	69.3	117–148
Ps FAM 2[c]	FAM-TAAGACTTGGTTGCATGATTTGAAAGAATCATT-TAMRA	68.5	132–164
Pt FAM 4[c]	FAM-TGAAAGAATCATTGTGATTAAGTATACGTGGCATTCT-TAMRA	70.0	150–186
StdLSUTex1	Texas Red-AGGGAGTATTCTAATGATTAACAGACCAACATCA-BHQ2	67.0	1,120–1,153

[a] Melting temperature; Tm was calculated at (2 μM) primer and (50 mM) monovalent cation concentration using the program MacVector (MacVector Inc., Cary, NC).

[b] For locations, *Puccinia graminis* sequence AY114289 was used for all primers, Pg FAM 1, and StdLSUTex1; *P. recondita* sequence DQ417420 was used for Pr FAM 2; *P. striiformis* sequence DQ417393 was used for Ps FAM 2; and *P. triticina* sequence AY187087 was used for Pt FAM 4.

[c] Pg FAM 1 = *P. graminis* probe, Pr FAM 2 = *P. recondita* probe, Ps FAM 2 = *P. striiformis* probe, and Pt FAM 4 = *P. triticina* probe.

sequence differences between the four *Puccinia* spp. Of the roughly 260- to 270-bp region of ITS1, all probes fell into a 97-bp region starting at base pair 47 through base pair 144 (Table 2). This region was devoid of polynucleotide repeats and, most importantly, was able to distinguish between the four rust pathogens (Fig. 2). Furthermore, among the diversity of collections within each fungal species, each FAM probe successfully detected only the rust fungal species it was designed to detect (Table 3).

An internal standard primer pair and probe combination was developed to verify the integrity of the PCR reaction and control against reporting false negative reactions. Like the ITS1 primer pair, the 28S primer pair, StdLSUF5a and StdLSUR2a, amplified only DNA from rust fungi, and did not amplify DNA from *U. maydis*, *F. graminearum*, or *T. aestivum* (Fig. 1B). However, the 28S primers did not amplify DNA from *Tranzschelia fusca* or *T. arthurii* (Table 3) and, therefore, has a more narrow range than the ITS1 primer pair. Although the StdLSUF5a and StdLSUR2a primer pair successfully amplified DNA, positive C_T values for all rust fungal DNA samples were obtained using the TexasRed probe whether the ITS1-specific FAM probe was positive or not (Table 3).

Assay sensitivity. The sensitivity of the real-time PCR assay was evaluated through serial dilutions of genomic DNA standards of known concentrations. Linearity of the standard curves extended over an at least 10^5-fold range of DNA concentrations with an r^2 of ≥0.99 for each assay (Fig. 3). As with conventional PCR, large amounts of DNA (≈100 ng/µl or higher) occasionally would result in a negative reaction (no 2nd derivative-C_T value) (data not

shown). The lower limits of detection, determined by serial dilutions of DNA, ranged from 0.053 to 0.53 pg for *P. graminis* DNA, 0.016 to 0.16 pg for *P. recondita* f. sp *secalis* DNA, 0.1 to 1.0 pg for *P. striiformis* DNA, and 0.067 to 0.67 pg for *P. triticina* DNA over multiple repetitions.

Assay specificity. To test possible interference of FAM probe specificity from DNA of more than one rust fungal species present in the same PCR assay, *P. graminis* and *P. triticina* DNA were combined in single-tube mixtures (0:100, 10:90, 25:75, 75:25, 90:10, and 100:0%) for a combined total of 500 pg µl^{-1} of fungal DNA. Mixtures were tested with either the *P. graminis* or the *P. triticina* FAM probe. Both probes showed a decrease in the C_T value as the concentration of the DNA specific to the probe increased (Fig. 4). C_T values for the *P. graminis* assay resulted in a regression line with an r^2 of 0.99 and a reaction efficiency of 96%. The results for the *P. triticina* assay were similar, with the regression line giving an r^2 of 0.90 and the reaction efficiency of 93%. Neither assay gave a positive C_T value for nontarget DNA (Fig. 4).

Specificity of primer pairs and probes was evaluated further through database searches and analysis of published sequences. BLASTN searches of nucleotide databases were done for the primer pairs and FAM probes for each of the four assays. Among the first 100 matches, the forward primer ITS1rustF10d showed 100% identity to *Puccinia* and *Uromyces* spp. and to some *Tilletia* spp. and other nonfungal species. However, the reverse primer ITS1rustR3c showed 100% identity only to *Puccinia* and *Uro-*

TABLE 3. Specificity of real-time polymerase chain reaction (PCR) assays for *Puccinia graminis*, *P. recondita*, *P. striiformis*, and *P. triticina*

Rust fungal species	Real-time PCR[a]						Rust fungal species	Real-time PCR[a]					
	I[b]	S[c]	Pg[d]	Pr[e]	Ps[f]	Pt[g]		I[b]	S[c]	Pg[d]	Pr[e]	Ps[f]	Pt[g]
P. graminis							*P. triticina*						
78-21-BB463	+	+	+	–	–	–	99NC28-1	+	+	–	–	–	+
75WA205A	+	+	+	–	–	–	93WA504	+	+	–	–	–	+
91MEX647	+	+	+	–	–	–	82TX176	+	+	–	–	–	+
72CAN1321A	+	+	+	–	–	–	82MN246	+	+	–	–	–	+
73VA441A	+	+	+	–	–	–	87NY678	+	+	–	–	–	+
93NY224B	+	+	+	–	–	–	00CA135	+	+	–	–	–	+
91KS60A	+	+	+	–	–	–	90KS285	+	+	–	–	–	+
91WA496A	+	+	+	–	–	–	00EGY38B	+	+	–	–	–	+
56SD37B	+	+	+	–	–	–	00EGY200C	+	+	–	–	–	+
84FL680	+	+	+	–	–	–	98EGY166C	+	+	–	–	–	+
74KS264A	+	+	+	–	–	–	98EGY151C	+	+	–	–	–	+
80MN633B	+	+	+	–	–	–	99SPN40AO	+	+	–	–	–	+
HSZ0802	+	+	+	–	–	–	03SPN24AO	+	+	–	–	–	+
HSZ0803	+	+	+	–	–	–	HSZ0745	+	+	–	–	–	+
HSZ0753	+	+	+	–	–	–	*P. andropogonis*						
HSZ0009	+	+	+	–	–	–	HSZ0261	+	+	–	–	–	–
P. recondita							HSZ0222	+	+	–	–	–	–
80WV271	+	+	–	+	–	–	HSZ0237	+	+	–	–	–	–
82WI47	+	+	–	+	–	–	HSZ0227	+	+	–	–	–	–
91TX9503	+	+	–	+	–	–	*P. bromina*						
HSZ0698	+	+	–	+	–	–	HSZ0663	+	+	–	–	–	–
ANK9958	+	+	–	+	–	–	HSZ0664	+	+	–	–	–	–
ANK9974	+	+	–	+	–	–	*P. arrhenathericola*						
P. striiformis							HSZ0659	+	+	–	–	–	–
PSH14	+	+	–	–	+	–	*P. triseti*						
PSH31	+	+	–	–	+	–	HSZ0700	+	+	–	–	–	–
PST29	+	+	–	–	+	–	*P. coronata*						
PST78	+	+	–	–	+	–	HSZ0425	+	+	–	–	–	–
52/99	+	+	–	–	+	–	*P. persistens*						
HSZ0722	+	+	–	–	+	–	HSZ0688	+	+	–	–	–	–
HSZ0724	+	+	–	–	+	–	*Tranzschelia arthurii*	+	–	–	–	–	–
							T. fusca	+	–	–	–	–	–

[a] A threshold (Ct) value of 30 was used; + = positive and – = negative.

[b] I = internal transcribed spacer 1 (ITS1) ITS1rustF10d and ITS1rustR3c primer pair amplifies ITS1 sequence of rDNA region; + = visible amplicon and – = no visible amplicon by gel electrophoresis.

[c] S = internal standard assay using StdLSUF5a and StdLSUR2a primer pair with TexasRed probe.

[d] Pg = ITS1rustF10d and ITS1rustR3c primer pair, and *P. graminis* FAM probe.

[e] Pr = ITS1rustF10d and ITS1rustR3c primer pair, and *P. recondita* f. sp. *secalis* FAM probe.

[f] Ps = ITS1rustF10d and ITS1rustR3c primer pair, and *P. striiformis* FAM probe.

[g] Pt = ITS1rustF10d and ITS1rustR3c primer pair, and *P. triticina* FAM probe.

myces spp. For the *P. graminis* species FAM probe, a 100% identity was found only to *P. graminis*, with the next closest sequence having 12 single-base-pair differences to homologous ITS1 DNA sequence. The same degree of specificity was found for the *P. recondita* f. sp. *secalis* FAM probe, with 100% identity only to that species and the next closest sequence also having 12 single-base-pair differences. However, BLASTN results showed fewer single-base-pair differences for the other two FAM probes. The *P. striiformis*-specific FAM probe showed a 1-bp difference to *P. monoica* homologous ITS1 DNA sequence and the related species *P. thlaspeos* and *P. consimilis*, and two single-base-pair differences to *P. coronata* f. sp. *avenae*, *P. allii*, *U. scillarum,* and *U. reichertii*. The *P. triticina* FAM probe showed only 1-bp difference to *P. sessilis*, but ≥9 single-base-pair differences to all other organisms.

Assay application. Real-time PCR analysis confirmed sample HSZ0887 as *P. graminis*, as well as *P. striiformis* for HSZ0886 and HSZ0885 (Table 4). Sample HSZ0882 was positively identified as *P. striiformis* by real-time PCR, which had conflicting results between the field observation and urediniospore morphological characteristics. Finally, two of the six samples were negative with all real-time PCR assays, except the internal controls indicating that these samples were rust fungi other than the four species tested. Recently, a *P. coronata* real-time PCR probe was developed, and these two samples tested positive (data not shown). In addition, the ITS region of all six samples was sequenced, and confirmed the real-time PCR results.

DISCUSSION

The objective of this study was to develop rapid real-time PCR assays to identify and detect four common cereal and grass rust pathogens, *Puccinia graminis*, *P. recondita* f. sp. *secalis, P. striiformis*, and *P. triticina*. Each assay was 100% specific and consistently detected DNA concentrations ≤1.0 pg. This is equivalent to less than five urediniospores (4). The highly sensitive, specific, and rapid assays described here, as well as the portability of some SmartCycler devices (31), make the assays ideal for detecting the four rust pathogens in the early stages of disease development, and can be developed for other rust fungi. This report is the first to describe detection and discrimination of these four rust pathogens using real-time PCR.

Sequence similarities and differences in the ITS1 region were useful in discriminating DNA of *P. graminis, P. recondita* f. sp. *secalis, P. striiformis*, and *P. triticina*. The large number of sequences we obtained from a variety of collections of each of the four rust pathogens increased our confidence in the specificity of the assays. Furthermore, BLASTN searches with the species-specific FAM probe sequences also have shown these assays to be specific for their intended target. White et al. (36) suggested that rDNA
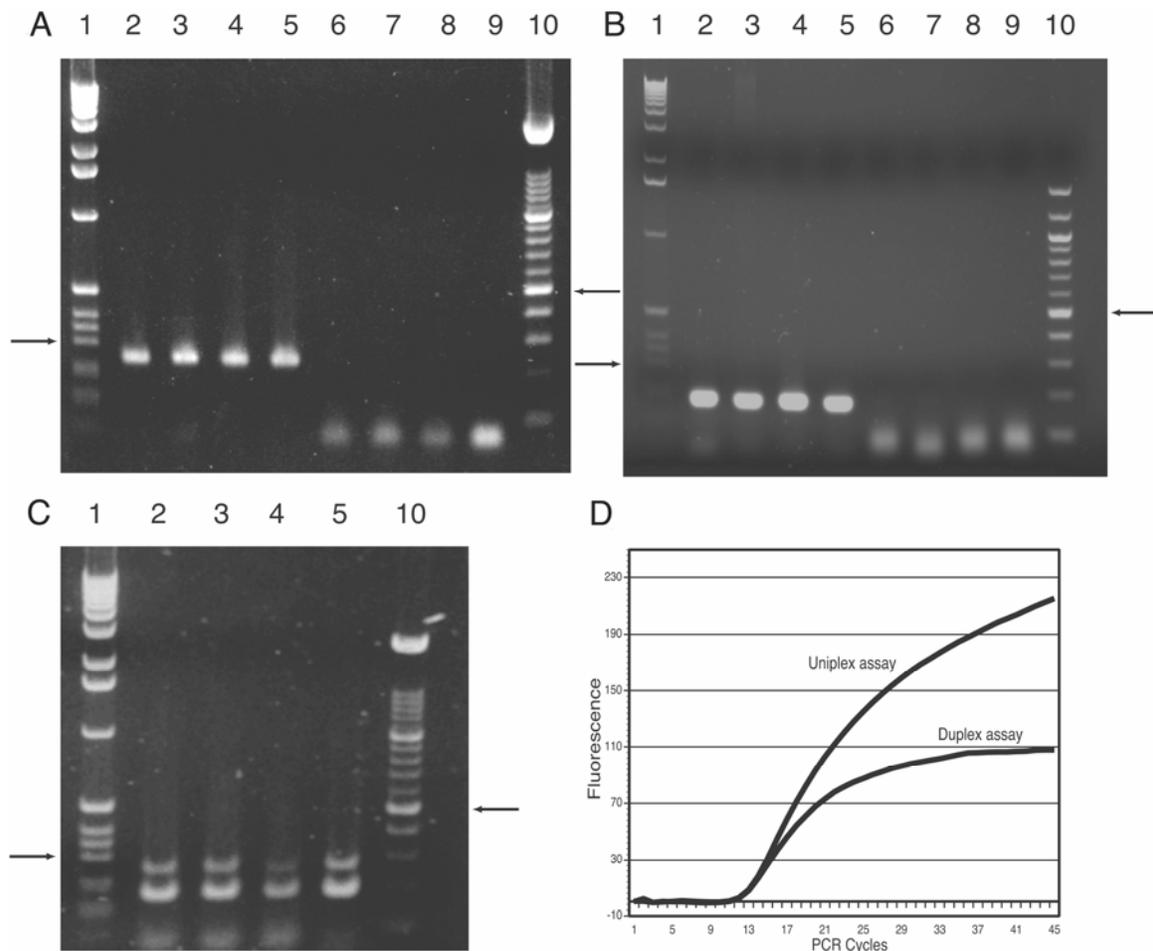

Fig. 1. Real-time polymerase chain reaction (PCR) amplification (1.0% agarose gels) of ribosomal DNA stained with ethidium bromide. **A,** internal transcribed spacer 1 (ITS1) region using the ITS1rustF10d/ITS1rustR3c primer pair; **B,** the 28S region using StdLSUF5a/StdLSUR2a primer pair; and **C,** duplex assay using both primer pairs. Lanes: 1, 1-kb molecular weight (MW) marker; 2, *Puccinia graminis*; 3, *P. recondita*; 4, *P. striiformis*; 5, *P. triticina*; 6, *Ustilago maydis*; 7, *Fusarium graminearum*; 8, *Triticum aestivum*; 9, no template control; and 10, 100-bp MW marker. Arrows on the left of each figure indicate the 296-bp band of the 1-kb MW marker and arrows on the right of each figure indicate a 500-bp band of the 100-bp MW marker. **D,** Amplification of *P. graminis* DNA by real-time PCR with ITS1 primer pair alone (uniplex assay) and in combination with 28S primer pair (duplex assay), both with the *P. graminis*-specific 6-carboxy-fluorescein probe. The reactions are equivalent to lane 2 in A and C, respectively.

could be useful for differentiating species because of the low evolutionary pressure on this area of the DNA that has resulted in its duplication in the genome. The use of ITS regions 1 and 2 for the production of specific primers has proven to be a successful strategy for developing diagnostic assays for many plant-pathogenic fungi (5,9,14).

The specificity of the assays for each rust fungal species held true for samples collected at diverse locations, times, and sources.

Fig. 2. Real-time polymerase chain reaction (PCR) duplex assays containing DNA from the four *Puccinia* spp., plus *Triticum aestivum*. Assay results in the internal transcribed spacer 1 (ITS1) column were amplified with the ITS1rustF10d/ITS1rustR3c primer pair along with 6-carboxy-fluorescein probes specific to each rust fungal species: **A,** *Puccinia graminis*; **B,** *P. recondita* f. sp. *secalis*; **C,** *P. striiformis*; and **D,** *P. triticina*. Each assay was specific to the intended rust fungal species. Assays in the 28S column were the internal standard assays duplexed with the adjacent ITS1 assay. Internal standard assays utilized the StdLSUF5a/StdLSUR2a primer pair along with the TexasRed probe for all assays. Only rust fungal DNA was amplified by the StdLSUF5a/StdLSUR2a primer pair.

Known isolates of *P. graminis* tested from Canada, Mexico, the Czech Republic, and throughout the United States from 1956 until the present all tested positive only with the *P. graminis*-specific assay. For the most closely related species, the leaf rust fungi *P. recondita* f. sp. *secalis* and *P. triticina* (formerly *P. recondita* f. sp. *tritici*), each assay was specific to the particular species, and did not detect other closely related leaf rust fungal species found on *Bromus, Arrhenatherum, Trisetum,* or *Elytrigia* grasses. These results suggest the FAM probes are specific within the *P. recondita* species complex for the two rust fungal species evaluated in this study. Furthermore, the use of leaf tissue samples provided additional support for assay specificity because any number of pathogens or saprophytes could have been present on leaf tissue samples along with the rust fungus of interest.

Individual FAM probes also were able to distinguish and quantify DNA from individual species from a mixture of species DNA. Therefore, the utility of the assays could be expanded to study competition between rust fungi on a single plant, or detecting and quantifying species from environmental samples (rain or air), where the host is unknown and a variety of organisms may be present. However, the linearity of the standard curves in the sample mixtures was more variable and slightly less efficient than assays using DNA of a single species. A PCR assay is 100% efficient if each cycle results in a doubling of the DNA concentration. However, few reactions are 100% efficient, and differences in PCR efficiency between samples being tested and an independent standard curve can greatly affect the quantitative measurement (6,17,22,23,25). Furthermore, the error in DNA quantification increased due to the logarithmic transformation in calculations (39).

Therefore, caution should be taken when surmising pathogen DNA concentration from a real-time PCR assay when mixtures of species may be present.

The amount of DNA as indicated in our assays may be a useful measure of fungal growth. However, DNA concentration may not relate directly to growth in fungal mass in plant tissues that contain various sizes of hyphae and sporophores as well as spores. Current methods, which rely on assays for glucosamine as an indication of chitin (1,18,35) or for the polyoles arabite and mannite that appear as constituents of urediniospores (3), also are limited by variation in fungal tissue and are more labor intensive. In the end, the accuracy, sensitivity, and ease of real-time PCR DNA quantification are a clear advance to current methods. Future research will be needed to correlate disease development with changes in DNA concentration. The assays developed in this study will be useful in these studies.

Equal concentrations of 28S and ITS1 primer pairs and both FAM and TexasRed probes resulted in similar C_T values for both the 28S and ITS1 assays. C_T values for the 28S assay indicated the amount and quality of rust fungal DNA present in negative ITS1 FAM probe reactions. In this study, we chose to keep primer pairs and probes in equal concentration to verify the presence of fungal DNA in a given sample and did not test duplex assays with lower 28S primer pair concentrations. Therefore, the amount of time for the confirmation of a negative result was roughly the same as for positive reactions. Positive results on the SmartCycler for the 28S region (C_T values >0), as well as for the ITS1 region, always were consistent with those of an agarose gel run at the end of each reaction. Fluorescence in duplex assays was reduced rela-
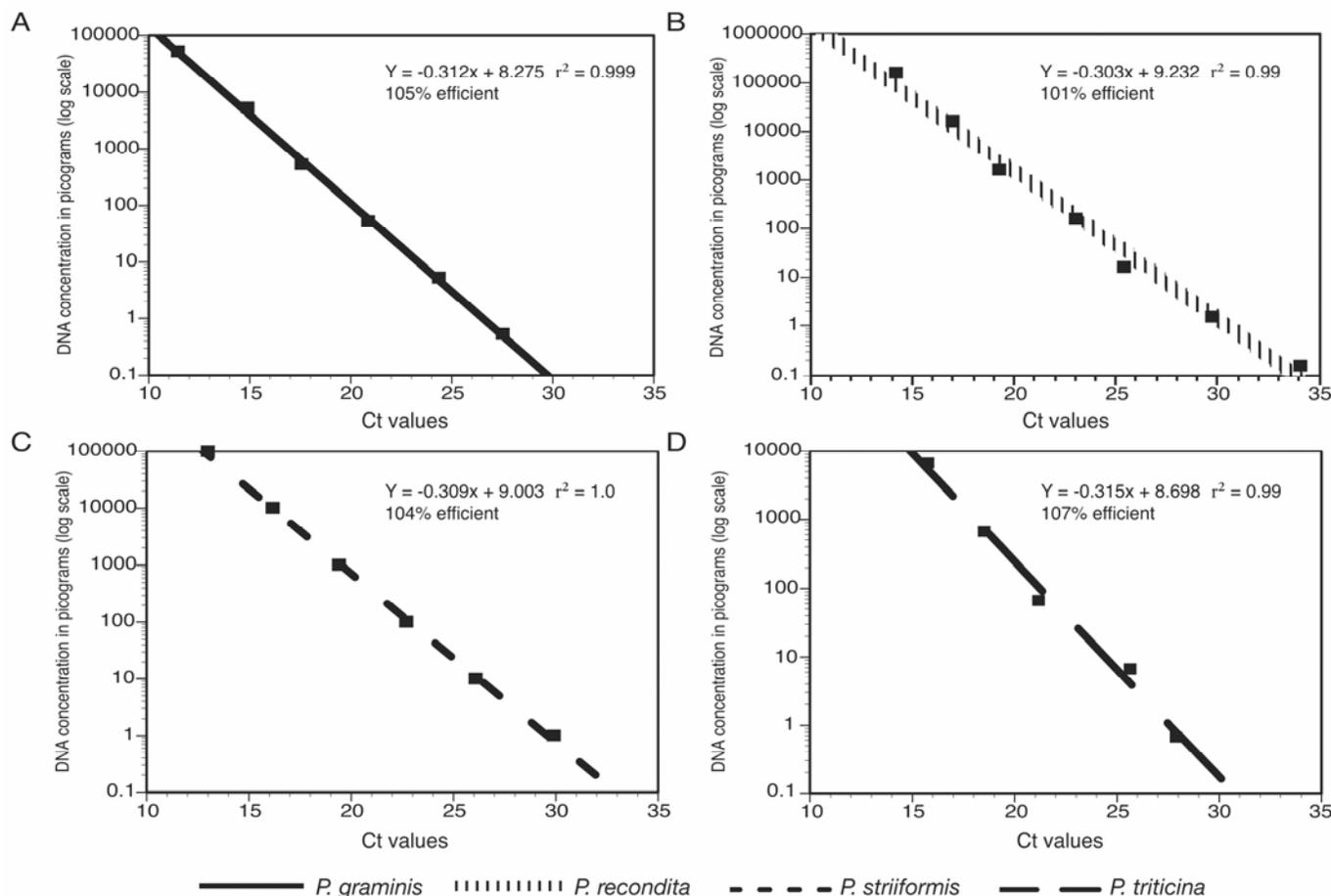

Fig. 3. Standard curves showing the straight line relationship between the log of known concentrations of DNA and the corresponding 2nd derivative threshold cycle (C_T) values from **A,** *Puccinia graminis*; **B,** *P. recondita* f. sp. *secalis*; **C,** *P. striiformis*; and **D,** *P. triticina*. Reaction efficiency was calculated as ($10^{(-slope)} - 1$) * 100.

tive to uniplex assays of the same DNA sample (Fig. 2D), suggesting that the amount of total amplicon was reduced (7,10). However, because the C_T value was not affected, the advantages of having primer pairs and probes in equal concentrations were not lost. These results further suggest that detection of target DNA in very low concentrations may be more reliable in uniplex assays.

We found that FAM probes need a minimum of three single-base-pair differences to maintain specificity among closely re-

lated rust fungal species within the confines of the PCR protocol. In this study, a preliminary *P. striiformis* probe having only two single-base-pair differences detected both *P. triticina* and *P. recondita* f. sp. *secalis*. To maintain the consistency in PCR conditions among assays, lengthening of the *P. striiformis* FAM probe to include an additional base pair difference made the *P. striiformis* FAM probe species specific. However, we also were successful in increasing specificity of the *P. striiformis* FAM probe with only two single-base-pair differences by increasing the
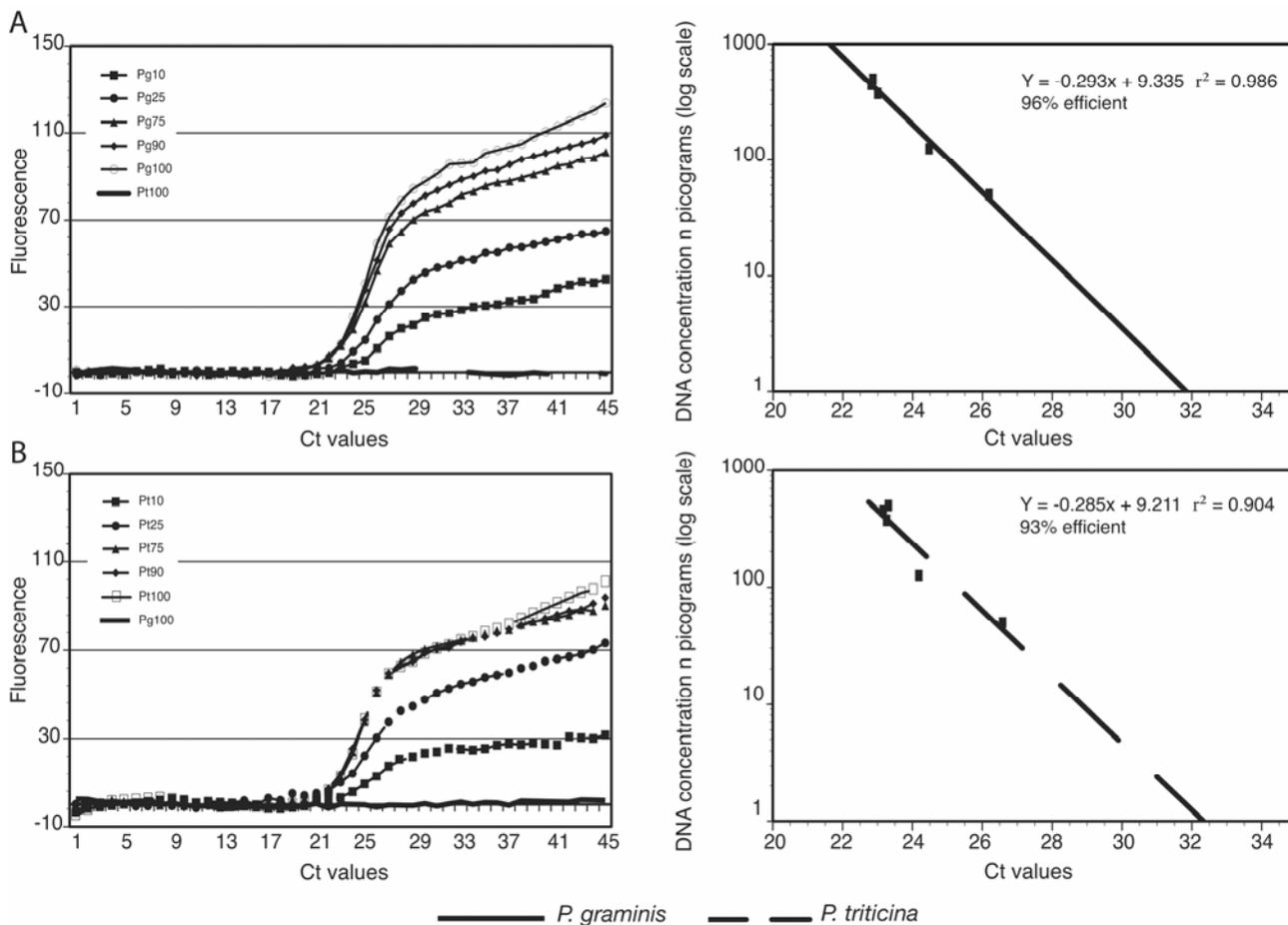
Fig. 4. Real-time polymerase chain reaction uniplex assays containing DNA from *Puccinia graminis* and *P. triticina* in mixtures totaling 500 pg of DNA at 100% (500 pg), 90% (450 pg; 50 pg), 75% (375 pg; 125 pg), 25% (125 pg; 375 pg), and 10% (50 pg; 450 pg) for both rust fungal species. 6-Carboxy-fluorescein probes specific to **A,** *P. graminis* and **B,** *P. triticina* were utilized in each mixture with corresponding standard curves to the right of each assay. Reaction efficiency was calculated as $(10^{(-\text{slope})} - 1) * 100$. C_T = threshold cycle.

TABLE 4. Use of real-time polymerase chain reaction (PCR) to identify unknown *Puccinia* spp. from pasture grasses

| | | | Real-time PCR | | | | | | | ITS DNA |
Sample	Field identification	Spore morphology	I[a]	S[b]	Pg[c]	Pr[d]	Ps[e]	Pt[f]	Pc[g]	Sequence
HSZ0882	*Puccinia striiformis*	*P. recondita*-like	+	+	−	−	+	−	−	*P. striiformis*
HSZ0883	*P. recondita*	*P. recondita*-like	+	+	−	−	−	−	+	*P. coronata*
HSZ0884	*P. striiformis*	*P. coronata*-like	+	+	−	−	−	−	+	*P. coronata*
HSZ0885	*P. striiformis*	*P. striiformis*-like	+	+	−	−	+	−	−	*P. striiformis*
HSZ0886	*P. striiformis*	*P. striiformis*-like	+	+	−	−	+	−	−	*P. striiformis*
HSZ0887	*P. graminis*	*P. graminis*-like	+	+	+	−	−	−	−	*P. graminis*

[a] I = internal transcribed spacer 1 (ITS1) ITS1rustF10d and ITS1rustR3c primer pair amplifies ITS1 sequence of rDNA region; + = visible amplicon and − = no visible amplicon by gel electrophoresis.

[b] S = internal standard assay using StdLSUF5a and StdLSUR2a primer pair with TexasRed probe.

[c] Pg = ITS1rustF10d and ITS1rustR3c primer pair, and *P. graminis* FAM probe.

[d] Pr = ITS1rustF10d and ITS1rustR3c primer pair, and *P. recondita* f. sp. *secalis* FAM probe.

[e] Ps = ITS1rustF10d and ITS1rustR3c primer pair, and *P. striiformis* FAM probe.

[f] Pt = ITS1rustF10d and ITS1rustR3c primer pair, and *P. triticina* FAM probe.

[g] Pc = ITS1rustF10d and ITS1rustR3c primer pair, and *P. coronata* FAM probe.

annealing temperature of the assay. Additionally, locked nucleic acid hybridization probes have been shown to distinguish single nucleotide polymorphisms using real-time PCR (13).

P. graminis and *P. striiformis* TaqMan assays were used successfully to distinguish stem rust and stripe rust on pasture grasses. It should be noted that the *P. striiformis* FAM probe does show close sequence similarity to several rust fungi, with 1-bp difference to *P. monoica* and the related species *P. thlaspeos* and *P. consimilis*, and two single-base-pair differences to *P. coronata* f. sp. *avenae*, *P. allii*, *U. scillarum,* and *U. reichertii*. The *P. striiformis* FAM probe did not show positive results when tested against *P. coronata*. f. sp. *avenae* DNA of ≈ 1.0 ng μl^{-1} or below. Weak false positive results occasionally were observed with higher DNA concentrations (data not shown). However, knowledge of the fungal DNA concentration and the host can eliminate some or all other rust fungal species that may show a false positive reaction.

PCR assay conditions and primers were optimized to DNA from as many rust fungi as possible, and not optimized for individual rust fungal species. To accomplish this, several primer pair combinations were analyzed for both the rust-fungus-specific primer pair and internal standard primer pair. The final primer pairs used for each assay were chosen based on low C_T values in uniplex and duplex assays and for compatibility with as many other rust fungi as possible. The flexibility built into the assay allows the assay to be adapted to other rust fungi simply by designing a new species-specific TaqMan probe. Variability outside the region in which the probes were designed can be used to generate primers to further differentiate rust fungal species to some extent. The goals of this study, however, were to maximize the versatility and simplicity by using standardized PCR conditions while maintaining assay specificity. Although knowledge of the host reduces doubt over possible false-positive results, application of the assay to spore identification from environmental samples, such as rain or air samples, may require greater primer specificity.

ACKNOWLEDGMENTS

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