A Multiplex Real-Time PCR Assay for the Detection of *Puccinia horiana* and *P. chrysanthemi* on Chrysanthemum

Jill E. Demers, Jo Anne Crouch, and Lisa A. Castlebury, Systematic Mycology and Microbiology Laboratory, United States Department of Agriculture, Agricultural Research Service, Beltsville, MD 20705

Abstract

Demers, J. E., Crouch, J. A., and Castlebury, L. A. 2015. A multiplex real-time PCR assay for the detection of *Puccinia horiana* and *P. chrysanthemi* on chrysanthemum. *Plant Dis.* 99:195-200.

Puccinia horiana, the cause of chrysanthemum white rust, is a regulated fungal plant pathogen in the United States, while *P. chrysanthemi*, the cause of chrysanthemum brown rust, is a widespread but less destructive pathogen. Accurate identification is essential to enforce quarantine measures, but the two species cannot be differentiated visually in the absence of mature spores or symptoms. A multiplex real-time PCR assay was developed to detect and discriminate between *P. chrysanthemi* and *P. horiana*. Species-specific hydrolysis probes labeled with different fluorescent dyes were designed based on the rDNA internal transcribed spacer region. Seven fresh samples and 270

herbarium specimens of chrysanthemum rust were tested with the assay with results confirmed using spore morphology. *P. horiana* and *P. chrysanthemi* were accurately detected from all fresh samples, and as little as 1 pg of template DNA was reproducibly detected. Of the herbarium specimens, 99% were positive for at least one species using the multiplex assay with 7% positive for both species. This multiplex assay can discriminate between *P. chrysanthemi* and *P. horiana* and provides an additional tool for identification of *P. horiana* to ensure appropriate application of quarantine measures.

Cultivated chrysanthemum (*Chrysanthemum* × *morifolium* Ramat.) is widely traded on both local and international scales, with annual wholesale production in the United States alone valued at \$145 million (22). Chrysanthemum plants are susceptible to a number of diseases that affect production yield and value. One of the most important diseases of chrysanthemum is white rust, caused by *Puccinia horiana* Henn. (Pucciniales), which is subject to quarantine and eradication in several countries, including the United States. The symptoms of white rust include chlorotic spots on the upper surface of the leaves, pustule formation mainly on the underside of the leaf (Fig. 1), and defoliation. Infection of chrysanthemum by *P. horiana* can be highly destructive, as 80 to 100% of plants can be infected during outbreaks for some susceptible cultivars (5,13). Furthermore, quarantine actions on infected plants can lead to complete crop loss. Another disease of chrysanthemum is brown rust, caused by *Puccinia chrysanthemi* Roze. In contrast with the extensive destruction caused by *P. horiana*, infection by *P. chrysanthemi* damages the cosmetic value of infected plants, affecting their marketability but only occasionally causing serious yield losses (20). Brown rust disease has been present in the United States for over 100 years (3) and is not currently regulated.

P. chrysanthemi and *P. horiana* can be distinguished by spore morphology (Fig. 1). *P. horiana* produces hyaline to pale yellow

teliospores that are 30 to 45 × 13 to 17 μm (14) and no urediniospores. *P. chrysanthemi* produces round, cinnamon-brown urediniospores that are 10 to 25 × 25 to 34 μm and dark brown teliospores that are 18 to 28 × 34 to 57 μm (4). The symptoms of brown rust and white rust are difficult to distinguish, as both cause chlorotic spots on the upper leaf surface and defoliation. *P. horiana* forms telia that are white to light brown due to the presence of hyaline teliospores and basidiospores, hence the name white rust. *P. chrysanthemi* produces brown uredinia, so it is known as brown rust. However, pustules caused by *P. horiana* may become darker with age and look similar to brown rust. Under certain conditions, *P. chrysanthemi* may form black telia, although these have been reported mostly in Japan, where the disease is known as black rust (4,15). Visual identification of either fungus is not possible in the latent stages of disease without sporulation (7,8,24) and for some resistant chrysanthemum cultivars on which sporulation does not occur (11).

In addition to *P. chrysanthemi* and *P. horiana*, the only other rust fungus described on *Chrysanthemum* × *morifolium* is *Phakopsora artemisiae* Hirats. f. (= *Uredo autumnalis*), reported exclusively in Asia (12). *Ph. artemisiae* is easily identified by its distinctive sub-epidermal teliospores and yellow urediniospores 22 to 30 × 17 to 25 μm in size. One more rust fungus, *Phakopsora compositarum*, has been reported on *C. indicum* (12) but is morphologically very similar to *Ph. artemisiae* and possibly synonymous. Several other rust fungi have been reported on *Chrysanthemum*, including *Puccinia aecidii-leucanthemi*, *P. heeringiana*, *P. leucanthemi*, *P. pyrethri*, and *P. tanacetii* (12,20). However, *Chrysanthemum* and related genera in the Anthemideae have undergone numerous taxonomic revisions (16,25), and these rust fungi do not affect plants in the genus *Chrysanthemum* as it is currently described.

The goal of this study was to create real-time PCR assays to detect both *P. chrysanthemi* and *P. horiana* to aid in the diagnosis and discrimination of these two chrysanthemum diseases. PCR-based detection assays have been previously developed for *P. horiana* using conventional PCR and real-time PCR (1,19). These real-time assays were shown to detect as little as 1 pg (19) or 5 fg (1) of *P. horiana* DNA and did not produce false positives with *P. chrysanthemi*, although both studies only included a single sample of *P. chrysanthemi*. The objective of this study was to develop a multiplex real-time PCR assay to simultaneously detect and discriminate

Corresponding author: L. Castlebury,
E-mail: Lisa.Castlebury@ars.usda.gov

*The e-Xtra logo stands for “electronic extra” and indicates that a supplementary table and two supplementary figures are available online.

Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the United States Department of Agriculture. USDA is an equal opportunity provider and employer.

Accepted for publication 28 August 2014.

<http://dx.doi.org/10.1094/PDIS-06-14-0632-RE>

This article is in the public domain and not copyrightable. It may be freely reprinted with customary crediting of the source. The American Phytopathological Society, 2015.

between *P. horiana* and *P. chrysanthemi* in fresh or dried specimens of chrysanthemum.

Materials and Methods

Fungal specimens and DNA extraction. Specimens of rust fungi used in this study are listed in Supplementary Table 1 and consisted of 187 herbarium accessions of *Puccinia chrysanthemi* and 83 accessions of *P. horiana* on *Chrysanthemum* spp. These collections were made from 30 countries between 1889 and 2004. Ten specimens of *Ph. artemisiae*, 18 additional *Puccinia* spp. on related host genera, and five non-target *Puccinia* spp. were also included. Four fresh specimens of *P. chrysanthemi* were collected in the mid-Atlantic region of the United States in October and November 2013 and identified microscopically using a Zeiss Axio-plan2 microscope with measurements made using Zeiss Axio-Vision 4.8.2 software. Asymptomatic leaves were collected from two *Chrysanthemum* sp. plants during August and November 2013, and leaves with spots but no sporulation were collected from three plants.

Fig. 1. Sori and spores of *Puccinia horiana* and *P. chrysanthemi*. **A**, *P. horiana* telia; **B**, *P. horiana* teliospore; **C**, *P. chrysanthemi* telia; **D**, *P. chrysanthemi* teliospore; **E**, *P. chrysanthemi* uredinia; **F**, *P. chrysanthemi* urediniospores. Scale bars = 20 µm.

To extract fungal genomic DNA, a small piece of leaf tissue with sori (approximately 0.25 to 1 cm²) was excised and manually ground using a micropestle in a 1.5-ml microcentrifuge tube with 0.5 mm diameter glass beads (BioSpec Products, Bartlesville, OK). Ground tissue was incubated at 55°C overnight in DNA extraction buffer AP1 from the DNeasy Plant Mini kit (QIAGEN, Gaithersburg, MD) with 400 µg RNase. The remaining steps of the extraction were carried out according to the manufacturer's instructions using the QIAcube automated sample prep machine (QIAGEN), and DNA was eluted in 100 µl buffer. DNA was extracted from asymptomatic leaves with the same method using approximately 2 cm² leaf material. For the fresh *P. chrysanthemi* specimen BPI 892937, which was used as a positive control, telia were scraped off of the leaves to minimize the amount of host DNA in the sample. Telia were ground in liquid nitrogen and DNA was extracted using the OmniPrep kit according to the manufacturer's instructions (G-Biosciences, St. Louis, MO). DNA was further purified using the Genomic DNA Clean and Concentrator kit (Zymo Research, Irvine, CA). DNA from fresh *P. horiana* specimens was provided by Doug Luster, from *P. horiana* spores harvested from infected chrysanthemum plants grown in containment at the USDA-ARS, Ft. Detrick, MD facility. DNA concentrations used for real-time PCR standard curves were measured using the Qubit dsDNA assay (Life Technologies, Grand Island, NY).

Real-time PCR assay development. PCR primers and probes for real-time PCR (Table 1) were developed using the software Beacon Designer 8.0 (PREMIER Biosoft, Palo Alto, CA) based on *P. chrysanthemi* isolate R81G-10 rDNA internal transcribed spacer (ITS) sequence (GenBank accession no. EU014038) (1) and *P. horiana* isolate Ph308 ITS sequence (EU013968) (1). ITS1 sequences from *P. chrysanthemi* (EU014034 to EU014038, EU400585, HQ201314 to HQ201322), *P. horiana* (EU013968, EU013972, EU013976, EU013999, EU014001, and EU014030), *P. tanacetii* (HQ201323 to HQ201325), and an unnamed *Puccinia* sp. from *Chrysanthemum* sp. (HQ201328 to HQ201331) were aligned using CLC Genomics Workbench 6.0.5 (CLC bio, Cambridge, MA), and hydrolysis probes were designed that bind to regions specific to *P. chrysanthemi* or *P. horiana*. Multiple ITS sequences have been reported in *P. horiana* (2), so primer binding sites were chosen that are present in all previously sequenced ITS clones. On the 5' end, the *P. chrysanthemi* CBR1 probe was labeled with 6-carboxyfluorescein (6-FAM) fluorescent dye and the *P. horiana* CWR2 probe was labeled with hexachloro-fluorescein (HEX) fluorescent dye. Both probes were labeled with Black Hole Quencher 1 on the 3' end (Sigma-Aldrich, Saint Louis, MO) and both contained four locked nucleic acids, which are bases with a modified backbone that increase probe specificity. A previously developed real-time PCR diagnostic assay for *P. horiana* (1) was also tested, using the primer pair Ph263F/Ph264R and the probe Ph706, labeled with 5' HEX, an internal ZEN quencher, and a 3' Iowa Black quencher. An additional real-time PCR assay was designed for an unnamed *Puccinia* sp. reported by Alaei et al. (1) on specimens of *Artemisia tridentata* and *Chrysanthemum* sp. based on the ITS1 region (HQ201331) and used to screen all samples. Primers and probes were synthesized by Integrated DNA Technologies (Coralville, IA).

Real-time PCR reaction mixtures contained 1X LightCycler 480 master mix (Roche, Indianapolis, IN), 1.0 µM of each primer, and

Table 1. Sequences of the primers and probes for real-time PCR amplification, with locked nucleic acids in the probes indicated with capital letters

Primers and probes		
<i>Puccinia chrysanthemi</i>		
Forward primer	CBR1F	TGGCTCAAATTTTATTAATTTAAAC
Reverse primer	CBR1R	GTTGCTTTTACTTGATTGAAA
Probe	CBR1	(5'-6FAM) tgaTtgTatGacGcgc (3'-BHQ1)
<i>Puccinia horiana</i>		
Forward primer	CWR2F	GCAGAAGGATCATTATTAAAAGA
Reverse primer	CWR2R	GGGTAATGTAACATTTCTTAGTAAA
Probe	CWR2	(5'-HEX) actTtaTtgTggCttgacc (3'-BHQ1)

0.2 µM probe, in 24 µl total volume. All reactions were replicated at least three times. Amplification was performed in white 96-well plates (Roche) in a LightCycler 480 II (Roche). The reaction cycle consisted of 95°C for 5 min followed by 55 cycles of 95°C for 10 s and the annealing temperature for 20 s, followed by 40°C for 30 s, with the annealing temperatures 50, 55, and 60°C tested and 55°C selected as optimal. Reaction threshold values were calculated with the second derivative method using Roche LightCycler 480 software (version 1.50.39). Assay efficiencies were calculated by constructing standard curves from 10-fold DNA dilutions (10 ng to 1 × 10⁻⁴ ng) from the DNA of *P. chrysanthemi* BPI 892937 or *P. horiana* specimen CT11-4.

All samples were tested using the *P. chrysanthemi* and *P. horiana* CBR1 and CWR2 real-time assays in combination. Duplexed CBR1/CWR2 reaction conditions were similar to those used for the single assay reactions, except that the reaction mixture consisted of 1× LightCycler 480 master mix, 1.0 µM of each of the four primers, 0.2 µM of each probe, and 1 µl undiluted DNA in a 12 µl reaction. All sets of reactions included a water-only negative control and positive controls of 1 ng of DNA from *P. chrysanthemi* BPI 892937 DNA and 1 ng of DNA from *P. horiana* CT11-4. For the herbarium DNA, different DNA dilutions (7×, 4×, 1×, 1:10, and 1:100 dilutions) were tested using three *P. chrysanthemi* specimens identified in preliminary tests as having high-, medium-, and low-quality DNA extracts based on crossing point (Cp) values for the CBR1 assay. One microliter of DNA extract was used for the freshly collected samples and asymptomatic leaves. The ability of the multiplex assay to detect mixed infections was tested on mixed DNA consisting of 1 ng DNA from *P. chrysanthemi* BPI 892937 and 1 ng DNA from *P. horiana* CT11-4.

Results

Assay development, amplification efficiency, and sensitivity limits. Real-time PCR hydrolysis probes for simultaneous detection of *P. chrysanthemi* and *P. horiana*, CBR1 and CWR2, were designed from the ITS1 regions of *P. chrysanthemi* isolate R81G-10 (EU014038) and *P. horiana* Ph308 (EU013968) (Fig. 2). The amplicon for the *P. chrysanthemi* CBR1 assay was 88 bp and the amplicon for the *P. horiana* CWR2 assay was 165 bp. Relative to the ITS sequence of *P. horiana* Ph308, 27 SNPs were situated in the *P. chrysanthemi* primers and probes (Fig. 2; forward primer CBR1F = 11; reverse primer CBR1R = 5; probe CBR1 = 11). Relative to the ITS sequence of *P. chrysanthemi* isolate R81G-10, 20 SNPs were situated in the *P. horiana* primers and probes (Fig. 2; forward primer CWR1F = 3; reverse primer CWR1R = 12; probe CWR1 = 5). BLASTn searches using the probe sequences as a query of NCBI GenBank showed that *P. chrysanthemi* sequences were the only fungal or plant sequences in GenBank with 100% identity to the probe CBR1 and the only close match in the Pucciniales to CBR1. The only matches for the sequence of the probe CWR2 were *P. horiana* and *Puccinia boroniae*, found only in Australia on *Boronia* spp., which does not have a sequence matching the CWR2R primer binding site.

Assays were tested with annealing temperatures at 50, 55, and 60°C. Fifty-five degrees was chosen as the best temperature to combine the two assays, as DNA amplified at lower cycle numbers with the CBR1 assay at 50 and 55°C than at 60°C, but more effi-

cient reactions occurred for the CWR2 assay at 55 and 60°C than at 50°C. Standard curves at 55°C were linear for 10 ng to 1 × 10⁻³ ng for both assays (*P. chrysanthemi* $r^2 = 0.991$; *P. horiana* $r^2 = 0.985$; Fig. 3). Crossing point (Cp) values for the *P. chrysanthemi* CBR1 assay ranged from 21.52 (10 ng) to 39.67 (1 pg), while Cp values for the *P. horiana* CWR2 assay ranged from 23.9 (10 ng) to 40.2 (1 pg). Amplification also occurred with 1 × 10⁻⁴ ng of DNA for both assays, but Cp values were not consistent across replicates. Multiplex CBR1/CWR2 reactions containing the same amount of template DNA yielded similar Cp values as for the individual assays (Fig. 3) (Wilcoxon rank sum test, $P = 0.37$). For 1 ng of template DNA, Cp values for CBR1 and CBR1/CWR2 were respectively 25.62 and 26.53, and values for CWR2 and CBR1/CWR2 were respectively 27.65 and 27.45. A previously published real-time assay for *P. horiana* (1) was tested in a multiplex reaction in conjunction with the CBR1 assay; however, late cycle low fluorescence levels barely detectable above the baseline were observed (Supplementary Fig. 1). An additional probe for *P. horiana* detection designed for the same region but with a higher melting point also generated low fluorescence and late cycle results.

The ability of the CBR1/CWR2 assays to detect mixed infections was tested by mixing 1 ng of pure DNA each from *P. chrysanthemi* BPI 892937 and *P. horiana* CT11-4. In the multiplex CBR1/CWR2 reaction containing both BPI 892937 and CT11-4 DNA, detection of *P. chrysanthemi* BPI 892937 was almost identical (Wilcoxon rank sum test, $P = 0.1$) to detection achieved from 1 ng of BPI 892937 DNA alone (Cp = 26.70 for only BPI 892937 DNA, Cp = 28.08 for BPI 892937 DNA mixed with CT11-4 DNA), but amplification of CT11-4 produced lower fluorescence levels than with 1 ng of *P. horiana* DNA alone, although with similar Cp values (Cp = 28.50 for only CT11-4 DNA, Cp = 28.39 for CT11-4 DNA mixed with BPI 892937 DNA; Supplementary Fig. 2) (Wilcoxon rank sum test, $P = 0.7$).

In addition to the *P. chrysanthemi* and *P. horiana* samples used for standard curve generation, the multiplex CBR1/CWR2 assay was tested using sori from three recently collected *P. chrysanthemi* samples and one recently collected *P. horiana* sample. In all cases, the target fungi were detected using the CBR1/CWR2 assay, with Cp values for the *P. chrysanthemi* samples between 26 and 29 cycles, corresponding to approximately 1 to 5 ng/µl of *P. chrysanthemi* DNA extracted per sample, and a Cp value of 30 cycles for a leaf infected with *P. horiana*, corresponding to 0.2 ng/µl of extracted *P. horiana* DNA. Positive results were obtained with the CBR1/CWR2 assay for a fresh sample of *P. horiana* extracted from spores, and the results were similar for those obtained for the control sample *P. horiana* CT11-4. The assay was negative for leaves collected from two asymptomatic chrysanthemum plants. Leaves from three chrysanthemum plants with leaf spots but no obvious signs of *Puccinia* species were tested, and one plant was positive for *P. chrysanthemi*, with 0.04 ng/µl of *P. chrysanthemi* DNA detected.

Specimen screening. The CBR1/CWR2 assay was used to screen 270 herbarium specimens identified as *P. chrysanthemi* or *P. horiana*. DNA was extracted from a mixture of fungal and host tissue. The optimum DNA concentration used in the assay was determined using three *P. chrysanthemi* DNA extractions, assigned to three broad quality categories (high, medium, and low quality)

Fig. 2. Alignment of the ITS DNA sequences of *Puccinia chrysanthemi* and *P. horiana* showing the CBR1/CWR2 primer and probe binding sites highlighted in gray.

based on Cp values with preliminary testing with the CBR1 assay, then assessed using the CBR1/CWR2 multiplex assay with 1 µl of 7×, 4×, 1×, 1:10, and 1:100 dilutions. DNA concentrations above 1× did not decrease Cp values for low- and medium-quality DNA. High-quality DNA exhibited the lowest Cp values at 4× concentration, and diluting the DNA extract increased the Cp values for all extracts tested. All herbarium samples were therefore evaluated with 1 µl of undiluted DNA extract as the template.

Ninety-four percent of the herbarium samples had Cp values between 27 and 45, 5% (14 specimens) had values between 45 and

55, and 1% (2 specimens) did not produce fluorescence, even after 55 cycles. Target DNA was detected in specimens dating as far back as 1889. Although the actual amount of *Puccinia* DNA extracted from herbarium samples could not be measured directly using spectrophotometry or fluorometry instruments due to the presence of host DNA, based on the CBR1 and CWR2 standard curves, DNA of the target pathogens was present at levels ranging from 100 ng to 0.01 ng in 100 µl total extract. Visual examination of the two negative specimens showed signs of *P. horiana* or *P. chrysanthemi*, but only scant material was available. Amplicons

Fig. 3. Standard curves showing the correlation of the crossing point cycle with the log concentration of DNA for a 10-fold dilution series from 10 ng to 1 pg of DNA for **A**, *Puccinia chrysanthemi* using the CBR1 real-time PCR assay, **B**, *Puccinia chrysanthemi* using the CBR1/CWR2 real-time PCR assay, **C**, *Puccinia horiana* using the CWR2 real-time PCR assay, and **D**, *Puccinia horiana* using the CBR1/CWR2 real-time PCR assay. **E** and **F**, Fluorescence history of multiplex CBR1/CWR2 real-time PCR reactions (gray lines) compared to CBR1 real-time PCR reactions and CWR2 real-time PCR reactions (black lines). All reactions include 1 ng template DNA. **E**, detection of the *P. chrysanthemi* CBR1 probe (6FAM). **F**, detection of the *P. horiana* CWR2 probe (HEX). **G** and **H**, Fluorescence history of multiplex CBR1/CWR2 real-time PCR reactions showing **G**, the lack of amplification of 10 ng of DNA of *Puccinia horiana* CT11-4 by the CBR1 assay and **H**, the lack of amplification of 10 ng of DNA of *P. chrysanthemi* BPI 892937 by the CWR2 assay. Gray lines: *P. horiana* CT11-4, black lines: *P. chrysanthemi* BPI 892937.

were not observed for these two specimens using agarose gel electrophoresis.

Eight herbarium specimens morphologically identified by their original collectors as *P. chrysanthemi* and 12 specimens identified by collectors as *P. horiana* produced positive results for both the CBR1 and CWR2 probes. Four of the eight *P. chrysanthemi* specimens and five of the 12 *P. horiana* specimens where two detections were made using the CBR1/CWR2 assay were observed microscopically to be infected with both rust fungi, while only one fungus was visually observed on the other 11 samples. Three specimens originally identified as *P. chrysanthemi* produced negative results using the *P. chrysanthemi* CBR1 assay and positive results using the *P. horiana* CWR2 assay. These three specimens were confirmed microscopically as *P. horiana*.

Assay specificity. Using DNA from known samples, the *P. horiana* CWR2 assay did not detect *P. chrysanthemi* and the *P. chrysanthemi* CBR1 assay did not detect *P. horiana*, even when tested against 10 ng of pure DNA (Fig. 3). Other species of rust fungi were tested with the multiplex CBR1/CWR2 assay, including an uncommon species known to infect *Chrysanthemum* (*Ph. artemisiae*), as well as 23 additional *Puccinia* spp. The *P. horiana* CWR2 assay produced negative results for all other tested species except *P. artemisiicola* on *Artemisia campestris*, *P. artemisiicola* on *A. stolonifera*, and *P. heeringiana* on *Tanacetum parthenium*. These three specimens were unmistakably different from *P. horiana* based on morphology, with both species producing reddish-brown telia with pale brown teliospores. The teliospores for *P. artemisiicola* were 44 to 71 × 20 to 33 µm and teliospores for *P. heeringiana* were 43 to 59 × 23 to 27 µm, all significantly larger than *P. horiana* teliospores. The *P. chrysanthemi* CBR1 assay was negative for all other species of rust fungi when combined with the *P. horiana* CWR2 assay. Very low levels of fluorescence, slightly above the baseline, were observed for *P. tanacetii* on *Tanacetum vulgare* and *P. pyrethri* on *Tanacetum corymbosum* with the multiplex assay, although these were not observed with the *P. chrysanthemi* CBR1 assay alone. Positive results were observed from the *P. chrysanthemi* CBR1 assay alone, but not from the multiplex CBR1/CWR2 assay, for *Puccinia similis* on *Artemisia tridentata* and *A. cana*, although detection of these specimens always occurred after cycle 45. Sequences for the *P. similis* specimens are available in NCBI GenBank (GU168942 and GU168943) and confirm they are very similar to the undescribed *Puccinia* species identified by Alaei et al. (1) with at least 99.6% sequence identity in the 5' end of the ribosomal large 28S subunit. Another real-time PCR assay, referred to as CBR2, was designed to detect this *Puccinia* sp. and was used to test all *P. chrysanthemi* and morphologically similar rusts. The CBR2 assay was positive for the two *P. similis* specimens but no others, including the *P. chrysanthemi* samples.

Discussion

P. horiana is a quarantine pathogen in the United States, and accurate detection of this fungus is important for effective containment and eradication efforts. This study provides a multiplex real-time PCR assay for the simultaneous detection of *P. chrysanthemi* and *P. horiana*, the two most common rust fungi on chrysanthemum.

The multiplex CBR1/CWR2 assay was effective across a wide range of DNA concentrations and could reproducibly amplify target DNA to a lower limit of 1 pg. The CBR1/CWR2 assay compares well to the two previously published real-time PCR assays designed to detect *P. horiana*, which were reported to detect 1 pg (19) or 5 fg (1) of *P. horiana* DNA. In addition to detecting *P. horiana*, the CBR1/CWR2 assay also detects *P. chrysanthemi*, a common pathogen that can affect the marketability of chrysanthemums and can be confused with *P. horiana*. An assay has been published (19) that uses different primer sets to detect both *P. chrysanthemi* and *P. horiana* but, as discussed by the author, is not appropriate for a multiplex assay because the probe sequence is not species-specific. The multiplex assay presented here will detect both *P. horiana* and *P. chrysanthemi* in one reaction.

Identification of *P. chrysanthemi* and *P. horiana* using the real-time PCR assay provided greater accuracy and sensitivity than visual identification alone. Three specimens originally identified as *P. chrysanthemi* in herbarium materials were found to be *P. horiana* based on our real-time PCR data and subsequent morphological re-examination. The multiplex CBR1/CWR2 assay detected both *P. chrysanthemi* and *P. horiana* in the same sample for 20 of the 270 herbarium specimens tested, demonstrating that infections by both rust fungi in one plant occasionally occur. In the multiplex CBR1/CWR2 assay, fluorescence levels for the *P. horiana* CWR2 probe are lower when both species are present. We suggest that chrysanthemum samples that are ambiguously positive (i.e., have low fluorescence levels or amplification after cycle 40) for *P. horiana* when *P. chrysanthemi* is also detected should be verified by using each of the two assays independently. Testing multiple pustules per leaf is also recommended in case both rust species are present.

Latent infections have been reported for both *P. chrysanthemi* and *P. horiana* (8,24), including systematic infection of overwintering plants by *P. horiana* (7). Our results suggest that real-time PCR can be used to detect these pathogens in the absence of sporulation. For example, the CBR1/CWR2 assay provided detection of *P. chrysanthemi* from one chrysanthemum sample exhibiting chlorotic leaf spots but lacking fungal spores. Furthermore, 11 specimens of diseased chrysanthemum, seven originally identified as *P. horiana* and four identified as *P. chrysanthemi*, generated positive assay results for both fungi, although only one fungus was observed microscopically.

P. chrysanthemi has undergone taxonomic changes and has at times been synonymized with morphologically similar fungi on related hosts, such as *P. absinthii*, *P. dracunculina*, *P. tanacetii*, and *P. pyrethri* (10). However, inoculation studies with *P. chrysanthemi* found that the fungus only causes disease on species of *Chrysanthemum* (15), and rusts on other hosts appear to be caused by other species (18). For example, a phylogenetic study by Alaei et al. (2) based on ITS sequence data showed that *P. chrysanthemi* forms a distinct clade within *Puccinia*, closely related to *P. tanacetii* on *Tanacetum* (Anthemideae) and an unnamed *Puccinia* sp. on *Chrysanthemum* sp. It is not clear if this unnamed *Puccinia* species causes disease on *Chrysanthemum sensu stricto* or on one of the chrysanthemum species now classified in a different genus in the Anthemideae, but an assay designed to detect this fungus was only positive for two specimens on *Artemisia* and was negative for all specimens on *Chrysanthemum*.

The present study used herbarium material from the U.S. National Fungus Collections, which allowed us to test specimens from a worldwide collection, likely to be representative of the genetic diversity in *P. horiana* and *P. chrysanthemi*. One hundred eighteen specimens were from Japan, the suspected origin of both *P. horiana* and *P. chrysanthemi*. The collection was also likely to be genetically diverse, as hosts included several species of *Chrysanthemum* and were collected from 1889 to 2004. The earliest specimen of *P. chrysanthemi* from the United States was from 1896 from the first reported outbreak in the United States in Fitchburg, MA (3). The CBR1/CWR2 assay detected each target fungus for all fresh samples and all herbarium samples, with the exception of one specimen morphologically identified as *P. horiana* and one specimen identified as *P. chrysanthemi* in which insufficient material was present to extract enough DNA for detection. The positive results obtained for this diverse collection suggest that the CBR1/CWR2 assay will work for all populations of these rust fungi.

Although using herbarium specimens allows for testing of a genetically diverse collection, DNA from herbarium specimens is often degraded and fragmented (21). Despite this degradation, DNA from herbarium material can be amplified, especially with real-time PCR, which is more sensitive and uses smaller amplicon sizes than most conventional PCR methods. For the chrysanthemum rust fungi, Cp values for DNA extracted from herbarium material were within the linear range of the assay but higher than

the values obtained for fresh material (usually 30 to 45 versus 26 to 31, respectively). Real-time PCR has been previously used with herbarium material to detect the corn rust pathogens *Puccinia polysora* and *P. sorghi*, (9), the turfgrass anthracnose fungus *Colletotrichum cereale* (6), and the causal agent of citrus bacterial canker *Xanthomonas citri* (17). Real-time PCR is therefore a useful method for studying herbarium collections, which can be a valuable source of information on the diversity and historical distribution of fungal species (23).

P. horiana is currently excluded from the United States when found on imported chrysanthemums and eradicated when found within the country. This assay can detect plants infected with *P. horiana* that need to be eradicated and help ensure that stock plants are free of *P. horiana*. The greater sensitivity and accuracy of the assay compared to visual diagnosis will furthermore be useful in detecting *P. chrysanthemi* and *P. horiana* before the onset of symptom development.

Acknowledgments

We thank Bonnie To for help with processing the chrysanthemum rust samples and assay development, Tunesha Phipps for technical support, and the U.S. National Fungus Collections. This work was funded by USDA-APHIS 2013 Farm Bill 10201 and a 2013 Friends of Agricultural Research – Beltsville student summer internship. J.E.D. is supported by a 2012 USDA-ARS Headquarters Post-doctoral Fellowship to L.A.C.

Literature Cited

- Alaei, H., Baeyen, S., Maes, M., Höfte, M., and Heungens, K. 2009. Molecular detection of *Puccinia horiana* in *Chrysanthemum × morifolium* through conventional and real-time PCR. *J. Microbiol. Meth.* 76:136-145.
- Alaei, H., De Backer, M., Nuytinck, J., Maes, M., Höfte, M., and Heungens, K. 2009. Phylogenetic relationships of *Puccinia horiana* and other rust pathogens of *Chrysanthemum × morifolium* based on rDNA ITS sequence analysis. *Mycol. Res.* 113:668-683.
- Arthur, J. C. 1900. Chrysanthemum rust. *Bulletin of the Indiana Agricultural Experiment Station* 85:143-150.
- Arthur, J. C. 1934. *Manual of the Rusts in United States and Canada*. The Science Press Printing Co., Lancaster, PA.
- Baker, J. J. 1967. Chrysanthemum white rust in England and Wales 1963-66. *Plant Pathol.* 16:162-166.
- Beirn, L. A., Clarke, B. B., and Crouch, J. A. 2014. Influence of host and geographic locale on the distribution of *Colletotrichum cereale* lineages. *PLoS One* 9(5):e97706.
- Bonde, M. R., Murphy, C. A., Bauchan, G. R., Luster, D. G., Palmer, C. L., Nester, S. E., Revell, J. M., and Berner, D. K. 2015. Evidence for systemic infection by *Puccinia horiana*, causal agent of chrysanthemum white rust, in chrysanthemum. *Phytopathology* 105:91-98.
- Campbell, C. E., and Dimock, A. W. 1955. Temperature and the geographical distribution of chrysanthemum rust. *Phytopathology* 45:644-648.
- Crouch, J. A., and Szabo, L. J. 2011. Real-time PCR detection and discrimination of the southern and common corn rust pathogens *Puccinia polysora* and *Puccinia sorghi*. *Plant Dis.* 95:624-632.
- Cummins, G. B. 1978. *Rust Fungi on Legumes and Composites in North America*. University of Arizona Press, Tucson, AZ.
- De Jong, J., and Rademaker, W. 1986. The reaction of *Chrysanthemum* cultivars to *Puccinia horiana* and the inheritance of resistance. *Euphytica* 35:945-952.
- Farr, D. F., and Rossman, A. Y. (Current) Fungal Databases, Systematic Mycology and Microbiology Laboratory, ARS, USDA. Online: <http://nt.ars-grin.gov/fungal-databases/>.
- Göre, M. E. 2008. White rust outbreaks on chrysanthemum caused by *Puccinia horiana* in Turkey. *Plant Pathol.* 57:786.
- Hennings, P. 1901. Einige neue japanische Uredineen. *Hedwigia* 40:25-26.
- Hiratsuka, N. 1957. Three species of chrysanthemum rusts in Japan and its neighbouring districts. *Beihefte zur Sydowia, Annales Mycologici, Series II* 1:34-44.
- Liu, P. L., Wan, Q., Guo, Y. P., Yang, J., and Rao, G. Y. 2012. Phylogeny of the genus *Chrysanthemum* L.: Evidence from single-copy nuclear gene and chloroplast DNA sequences. *PLoS ONE* 7:e48970.
- Marvrodieva, V., Levy, L., Gabriel, D. W. 2004. Improved sampling methods for real-time polymerase chain reaction diagnosis of citrus canker from field samples. *Phytopathology* 94:61-68.
- Newcombe, G. 2003. *Puccinia tanacetii*: specialist or generalist? *Mycol. Res.* 107:797-802.
- Pedley, K. F. 2009. PCR-based assays for the detection of *Puccinia horiana* on chrysanthemums. *Plant Dis.* 93:1252-1258.
- Punithalingam, E. 1968. *Puccinia chrysanthemi*. *CMI Descriptions of Pathogenic Fungi and Bacteria*. No. 175.
- Staats, M., Cuenca, A., Richardson, J. E., Vrieling-van Ginkel, R., Petersen, G., Seberg, O., and Bakker, F. T. 2011. DNA damage in plant herbarium tissue. *PLoS ONE* 6:e28448.
- USDA National Agricultural Statistics Service. 2009. *Census of Horticultural Specialties*. Vol. 3. Part 3.
- Verkley, G. J. M., Rossman, A., and Crouch, J. A. 2013. The role of herbaria and culture collections. In: *The Mycota VII: Systematics and Evolution*, Part B. D. J. McLaughlin, M. Blackwell, and J. W. Spatafora, eds. In press.
- Zandvoort, R., Groenewegen, C. A., and Zadoks, J. C. 1968. On the incubation period of *Puccinia horiana*. *Neth. J. Pl. Path.* 74:128-130.
- Zhao, H. B., Chen, F. D., Chen, S. M., Wu, G. S., and Guo, W. M. 2010. Molecular phylogeny of *Chrysanthemum*, *Ajania* and its allies (Asteraceae, Asteraceae) as inferred from nuclear ribosomal ITS and chloroplast *trnL-F* IGS sequences. *Plant Syst. Evol.* 284:153-169.