Molecular Analysis of Turfgrass Rusts Reveals the Widespread Distribution of *Puccinia coronata* as a Pathogen of Kentucky Bluegrass in the United States

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Abstract

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Over the past 10 years, rust diseases have become increasingly prevalent on certain cultivars of Kentucky bluegrass. This pattern suggests that new races or new species of rust fungi may have emerged. To test this hypothesis, 66 samples of turfgrass rust fungi collected from across the United States were evaluated based on sequences of the internal transcribed spacer (ITS)-5.8S rDNA region. Phylogenetic analysis revealed three species: *Puccinia coronata*, *P. graminis*, and *P. striiformis*, comprising 67, 28, and 5% of the samples, respectively. *P. coronata* was frequently found in association with Kentucky bluegrass, a host–pathogen relationship that has not been previously reported. Comparison of molecular analyses with the use of standard field

identification techniques—host association and pustule pigmentation—showed that 58% of the Kentucky bluegrass samples would have been incorrectly diagnosed using nonmolecular criteria. To avoid such misidentifications, a real-time polymerase chain reaction diagnostic protocol was developed for turfgrass-associated *P. graminis*, *P. coronata*, and *P. striiformis* using ITS sequences. Accurate, reproducible, species-specific identifications were made using as few as 50 to 150 urediniospores, even in mixed infections. This study represents the first DNA-based evaluation of turfgrass rust fungi and provides a quick and reliable sequence-based protocol as an alternative to less reliable field-based identification techniques.

Rust is a common fungal disease of cultivated turfgrass that can occur in all turf species and is found worldwide (40). Multiple fungi are responsible for rust symptoms in turf. In temperate regions, the most prevalent turf rust fungi are *Puccinia graminis* Pers. (causes stem rust), *P. striiformis* Westend. (stripe rust), *P. coronata* Corda (crown rust), and *P. recondita* Dietel & Holw. and *P. brachypodii* G.H. Otth (leaf rust) (40). Diseased grasses typically exhibit an unsightly orange-brown discoloration, resulting in aesthetically unattractive turf that is unmarketable in sod production and often unacceptable in athletic fields, home lawns, and golf courses. Over time, diseased areas may die out completely due to decreased photosynthetic rates and increased dark respiration initiated by the fungus (13). Although fungicides can be used to minimize disease by limiting urediniospore production, chemical control is costly and sometimes ineffective (40). As a result, resistance in turfgrass cultivars is used as an important mode of defense against rust pathogens.

Over the past 10 years, turfgrass breeders have documented a gradual shift in susceptibility to rust among Kentucky bluegrass (*Poa pratensis* L.) cultivars, particularly the ‘Midnight’ types (10). Once highly resistant to rust, these cultivars are now experiencing a greater incidence of disease. Resistance to rust in Kentucky bluegrass is not well understood, and traditional breeding methods

remain the standard approach for incorporating disease resistance in turfgrass hosts (10). As has been reported in other grass and cereal systems, turfgrass rust resistance is likely the result of a single dominant gene in the host that matches an avirulence gene in the pathogen (7,11,12,31,35), although different genes may be required for specific fungal races of rust, and other environmental or host factors may also be important (39). Breakdown of rust resistance in wheat (*Triticum aestivum* L.) typically signals the emergence of a new race (34), and these new phenotypes may emerge frequently in pathosystems where the sexual cycle can be completed. It has been suggested that the emergence of novel race pathotypes, as seen in the wheat rust pathosystem, may be responsible for the shift in cultivar susceptibility observed in Kentucky bluegrass turf (10), but this prediction has not yet been tested. Pathotype specificity has been demonstrated for different isolates of *Puccinia graminis* in perennial ryegrass (*Lolium perenne* L.) turf (37) and is suspected for isolates of *P. coronata* pathogenic to this species (28,38,41). However, these observations were strictly qualitative and, to date, rust pathogen genotyping has not been performed from any turfgrass hosts.

Unlike the cereal rust pathosystems, the cohort of fungal species that are responsible for rust diseases of turfgrasses have not been well studied. The last comprehensive review of rusts on turf-type grasses was completed nearly 30 years ago (18) and predated the use of molecular techniques. Currently, there are a total of 39 species of *Puccinia*, *Physopella*, and *Uromyces* that have been described on turfgrasses based on morphological distinctiveness (40). The number of accepted rust species varies between studies, because morphological characteristics and host plant association (40) are the primary mode of identifying turfgrass rusts, and interpretation of these characteristics can vary depending on the taxonomic treatment used. The use of morphology for rust fungus identification is complicated, because subtle differences such as teliospore size, shape, and pedicel type (33) can vary between closely related species. Moreover, in many instances, only urediniospores are present on the host, a less distinctly shaped spore type that makes accurate morphological identifications difficult and sometimes impossible.

As with morphology, the use of host plant associations to identify rust fungus species can also be problematic. Complete lifecycle

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cles are unknown for many graminicolous rust fungi, making it impossible to fully describe host ranges (3), and cross-infectivity between hosts has been reported in inoculation studies (36). Despite the uncertainty associated with the use of host identity and spore phenotype for rust fungus species identification, these techniques are routinely used in the field, because they are the only tools currently available to breeders and turfgrass managers.

Sequence analysis of the internal transcribed spacer (ITS) region has been used to differentiate between closely related rust fungi (4,5,9,17,23,45); however, to our knowledge, there has never been

a DNA-based evaluation of species causing rust diseases in turfgrass hosts. Therefore, our objectives were to use sequence-based phylogenetic analysis of the ITS-5.8S rDNA region to identify fungal species responsible for rust diseases in turfgrasses, and to test the prediction that the shift in rust susceptibility observed among Kentucky bluegrass cultivars in the field is the result of novel species or race associations. We surveyed a range of diseased turfgrass hosts to assess molecular variability of the ITS-5.8S region in rust fungi associated with several commercially important turfgrass species. As an alternative to using morphological

Table 1. Sources of rust fungal samples used in this study and GenBank accession numbers for the rDNA internal transcribed spacer-5.8S sequences

Isolate	Host species	Origin	Accession number	Reference ^a
<i>Puccinia coronata</i>				
CR-1	<i>Arrhenatherum elatius</i>	Czech Republic	DQ355443	Mycoscience
MNCL-1	<i>Avena sativa</i>	Minnesota	AY114290	Mycoscience
UK-2	<i>Avena</i> sp.	United Kingdom	GU598105	...
UK-5	<i>Avena</i> sp.	United Kingdom	GU598106	...
UKF-12	<i>Avena</i> sp.	United Kingdom	GU598109	...
SLV-1	<i>Bromus erectus</i>	Slovakia	DQ355449	Mycoscience
CR-2	<i>B. erectus</i>	Czech Republic	DQ355450	Mycoscience
MNCL-9	<i>B. inermis</i>	United States	DQ355448	Mycoscience
MNCL-10	<i>B. inermis</i>	United States	DQ355446	Mycoscience
MI-1	<i>Bromus</i> sp.	Michigan	GU598069	...
UKF-8	<i>Calamagrostis</i> sp.	United Kingdom	GU598107	...
UKF-10	<i>Calamagrostis</i> sp.	United Kingdom	GU598108	...
NJA-24	<i>Deschampsia</i> sp.	New Jersey	GU598078	...
MNCL-2	<i>Elytrigia repens</i>	Minnesota	DQ414723	Mycoscience
MNCL-3	<i>E. repens</i>	Minnesota	DQ414724	Mycoscience
MS-1	<i>Festuca arundinacea</i>	Mississippi	GU598056	...
NJA-66	<i>F. arundinacea</i>	New Jersey	GU598047	...
NJA-67	<i>F. arundinacea</i>	New Jersey	GU598048	...
NJA-9	<i>Festuca</i> sp.	New Jersey	GU598072	...
IR-1	<i>Holcus lanatus</i>	Ireland	DQ355444	Mycoscience
UO-1	<i>Hordeum vulgare</i>	Unknown	DQ355454	Mycoscience
CH-1	<i>Lolium perenne</i>	Chile	GU598061	...
CH-3	<i>L. perenne</i>	Chile	GU598062	...
CT-3	<i>L. perenne</i>	Connecticut	GU598059	...
MA-1	<i>L. perenne</i>	Massachusetts	GU598063	...
MA-2	<i>L. perenne</i>	Massachusetts	GU598064	...
MA-3	<i>L. perenne</i>	Massachusetts	GU598065	...
NJC-7	<i>L. perenne</i>	New Jersey	GU598079	...
NJCC-31	<i>L. perenne</i>	New Jersey	GU598083	...
NJCC-33	<i>L. perenne</i>	New Jersey	GU598084	...
CR-3	<i>L. perenne</i>	Czech Republic	DQ355441	Mycoscience
UK-3	<i>L. perenne</i>	United Kingdom	GU598049	...
UKF-13	<i>L. perenne</i>	United Kingdom	GU598110	...
UKF-14	<i>L. perenne</i>	United Kingdom	GU598111	...
NJNB-37	<i>Poa pratensis</i>	New Jersey	GU598091	...
NJCC-42	<i>P. pratensis</i>	New Jersey	GU598085	...
NJE-50	<i>P. pratensis</i>	New Jersey	GU598086	...
MEG-1	<i>P. pratensis</i>	Maine	GU598068	BPI881581
WY-1	<i>P. pratensis</i>	Wyoming	GU598112	...
NC-2	<i>P. pratensis</i>	North Carolina	GU598070	...
OH-3	<i>P. pratensis</i>	Ohio	GU598098	...
OH-4	<i>P. pratensis</i>	Ohio	GU598099	...
OH-5	<i>P. pratensis</i>	Ohio	GU598100	...
MA-4	<i>P. pratensis</i>	Massachusetts	GU598066	...
MA-5	<i>P. pratensis</i>	Massachusetts	GU598067	...
NJA-19	<i>P. pratensis</i>	New Jersey	GU598075	...
NJA-20	<i>P. pratensis</i>	New Jersey	GU598076	...
NJA-21	<i>P. pratensis</i>	New Jersey	GU598077	...
NJA-23	<i>P. pratensis</i>	New Jersey	GU598054	...
NJC-28	<i>P. pratensis</i>	New Jersey	GU598081	...
NJC-29	<i>P. pratensis</i>	New Jersey	GU598082	...
NJHP-32	<i>P. pratensis</i>	New Jersey	GU598090	...
NJT-4	<i>P. pratensis</i>	New Jersey	GU598092	...
OH-1B	<i>P. pratensis</i>	Ohio	GU598097	...
OH-8B	<i>P. pratensis</i>	Ohio	GU598052	...
MNCL-4	<i>Rhammus catharticus</i>	Minnesota	DQ355445	Mycoscience
MNCL-5	<i>R. catharticus</i>	Minnesota	DQ355447	Mycoscience
MNCL-6	<i>R. catharticus</i>	Minnesota	DQ355442	Mycoscience

(continued on next page)

^a In addition to literature citation numbers, "Mycoscience" refers to Mycoscience 47:130-136 and BPI numbers are voucher numbers for samples deposited in the U.S. National Fungus Collections (BPI).

characteristics and host plant association for turfgrass rust identification, these sequence data were then used to generate a real-time polymerase chain reaction (PCR) protocol that could be implemented by turfgrass breeding programs and diagnostic laboratories for the quick and accurate identification of turfgrass rust species.

Materials and Methods

Fungal samples. Eleven grass species symptomatic for rust disease and exhibiting visible urediniospores or teliospores were collected from 13 states in the United States, Australia, Canada, Chile,

and the United Kingdom. A complete list of the 66 fungal samples used in this study is presented in Table 1. Upon collection, samples were visually identified to species by urediniospore size, cell wall thickness, and shape and then photographed (*data not shown*). Tentative morphological identifications of the rust pathogens were made by evaluating 50 urediniospores from each sample. Teliospores were typically absent; however, when present, they were classified by pedicel length, overall shape, cell wall thickness, digitations, and size. Additionally, each sample was evaluated phenotypically for pustule color and orientation (e.g., presence of uredia parallel to leaf veins). Samples were then stored at 4°C until

Table 1. (continued from preceding page)

Isolate	Host species	Origin	Accession number	Reference ^a
MNCL-7	<i>R. catharticus</i>	Minnesota	DQ355452	Mycoscience
SLV-2	<i>R. catharticus</i>	Slovakia	DQ355451	Mycoscience
NJC-8	<i>Zoysia</i> sp.	New Jersey	GU598080	...
<i>Puccinia graminis</i>				
CR-4	<i>Anthroxanthum</i> sp.	Czech Republic	DQ417386	8
SW-1	<i>A. sativa</i>	Switzerland	DQ417391	8
SW-2	<i>Dactylis glomerata</i>	Switzerland	DQ417390	8
WI-1	<i>Elymus trachycalus</i>	Wisconsin	DQ417381	8
NJF-45	<i>F. arundinacea</i>	New Jersey	GU598087	...
NJH-48	<i>F. arundinacea</i>	New Jersey	GU598088	...
NJH-49	<i>F. arundinacea</i>	New Jersey	GU598089	...
OR-1	<i>F. arundinacea</i>	Oregon	DQ417385	8
OR-2	<i>L. perenne</i>	Oregon	DQ417384	8
UO-2	<i>L. perenne</i>	unknown	DQ355455	8
UO-3	<i>Lolium</i> sp.	unknown	DQ460726	25
MNCL-8	<i>Phleum pratense</i>	Minnesota	DQ417392	8
NJT-5	<i>Poa pratensis</i>	New Jersey	GU598093	...
NYPI-1	<i>P. pratensis</i>	New York	GU598095	...
NJGM-39	<i>P. pratensis</i>	New Jersey	GU598053	...
ONCA-1	<i>P. pratensis</i>	Canada	GU598103	...
NE-1	<i>P. pratensis</i>	Nebraska	GU598071	...
NC-1	<i>P. pratensis</i>	North Carolina	GU598070	BPI881582
OH-6	<i>P. pratensis</i>	Ohio	GU598101	...
OH-7	<i>P. pratensis</i>	Ohio	GU598102	...
MNM-1	<i>P. pratensis</i>	Minnesota	GU598058	...
NJA-53	<i>P. pratensis</i>	New Jersey	GU598057	...
NJA-54	<i>P. pratensis</i>	New Jersey	GU598060	...
NJA-17	<i>P. pratensis</i>	New Jersey	GU598073	...
NJA-18	<i>P. pratensis</i>	New Jersey	GU598074	...
OH-1A	<i>P. pratensis</i>	Ohio	GU598096	...
OH-8A	<i>P. pratensis</i>	Ohio	GU598051	...
SW-3	<i>P. pratensis</i>	Switzerland	DQ417389	8
KS-1	<i>Triticum aestivum</i>	Kansas	DQ417374	8
OH5-1	<i>T. aestivum</i>	Ohio	DQ417375	8
MX-1	<i>T. aestivum</i>	Mexico	DQ417377	8
OR-3	<i>T. aestivum</i>	Oregon	DQ417380	8
SW-4	<i>T. aestivum</i>	Switzerland	DQ417387	8
SD-1	<i>T. aestivum</i>	South Dakota	DQ417383	8
UO-4	<i>T. aestivum</i>	Unknown	AY114289	Mycoscience
<i>Puccinia striiformis</i>				
MNC-1	<i>Berberis chinensis</i>	Minnesota	GU382672	8
MNC-5	<i>B. chinensis</i>	Minnesota	GQ457305	27
CR-5	<i>D. glomerata</i>	Czech Republic	DQ417403	27
WA-1	<i>Hordeum vulgare</i>	Washington	DQ417408	8
CA-1	<i>H. vulgare</i>	California	DQ417402	8
PA-2	<i>L. perenne</i>	Pennsylvania	GU598104	...
NYH-4	<i>L. perenne</i>	New York	GU598094	BPI881580
AU-1	<i>Poa annua</i>	Australia	GU598050	...
GA-1	<i>P. pratensis</i>	Georgia	DQ417407	8
MNC-2	<i>T. aestivum</i>	Minnesota	GU382671	5
MNC-3	<i>T. aestivum</i>	Minnesota	GU382673	5
MNC-4	<i>T. aestivum</i>	Minnesota	DQ417394	8
WA-2	<i>T. aestivum</i>	Washington	DQ417395	8
DNM-1	<i>T. aestivum</i>	Denmark	DQ417397	8
DNM-2	<i>T. aestivum</i>	Denmark	DQ417404	8
TX-1	<i>T. aestivum</i>	Texas	DQ417405	8
TX-2	<i>T. aestivum</i>	Texas	DQ417406	8
<i>Puccinia hordei</i>				
UO-5	<i>H. vulgare</i>	Unknown	AY187089	5
<i>P. recondita</i>				
UO-6	<i>H. vulgare</i>	Unknown	EU014045	4

used for molecular analyses. Representative voucher specimens were submitted to the U.S. National Fungus Collections (BPI) for each of the three *Puccinia* spp. sampled (BPI1881580–BPI1881582).

DNA manipulations. Spores were directly harvested from rust-infected tissue, placed in a 2-ml microcentrifuge tube containing five or six 2-mm glass beads (BioSpec Products, Bartlesville, OK), and shaken in a BioSpec bead-beater on the medium setting for 6 min. Extraction buffer (2× STE [1× = 100 mM NaCl; 50 mM Tris, pH 8.0; and 1 mM EDTA], 2% sodium dodecyl sulfate, and 2% β-mercaptoethanol) was added to create a slurry that was mixed with 1× volume phenol. After ethanol precipitation of the supernatant, pelleted DNA was resuspended in Tris-EDTA (TE) buffer (1 M Tris [pH 8.0] and 0.5 M EDTA) and incubated at 37°C with RNaseA (1 μg/ml) for 30 min. After an additional phenol:chloroform extraction and ethanol precipitation, pellets were resuspended in TE and DNA concentration and purity was determined using a NanoDrop 1000 Spectrophotometer (Wilmington, DE).

PCR primers ITS4 and ITS5 (44) were used to amplify the complete nuclear ribosomal ITS1, 5.8S, and ITS2 regions, including partial flanking regions of the 18S and 28S rDNA. All samples were amplified in a GeneAmp Thermocycler (Applied Biosystems, Foster City, CA) using cycling conditions previously described (16). Amplicons were excised from 0.8% agarose gels, purified using the GeneClean III Kit (QBiogene, Irvine, CA), ligated into pGEM-T Easy vectors (Promega Corp., Madison, WI), and transformed into α-Select Gold Efficiency competent cells (BioLine, Taunton, MA) to account for (i) mixed rust species that may be present in the samples or (ii) dikaryon genome heterogeneity. At least three clones from each isolate were selected for sequencing. Insert sequences were generated from plasmid DNA purified with the Qiaprep Spin Miniprep Kit (Qiagen, Valencia, CA). Sanger sequencing was performed on both strands by GeneWiz, Inc. (South Plainfield, NJ) from the SP6 and T7 primer sites located on the vector. Sequences were assembled using Lasergene Sequence Analysis Software (DNASTAR, Inc., Madison, WI). Sequences were deposited in National Center for Biotechnology Information GenBank (<http://www.ncbi.nlm.nih.gov/>) (accession numbers GU598047 to GU598112).

Phylogenetic analyses. Two phylogenetic analyses were performed using the ITS sequences generated from this study, along with 49 additional sequences from grass hosts obtained from GenBank (Table 1). Only sequences associated with publication in a peer-reviewed journal were chosen for inclusion in this study. *P. hordei* and *P. recondita* were used as outgroup taxa for an analysis inclusive of all sampled taxa (1,45). *P. graminis* was used as the outgroup for the analysis of a dataset composed entirely of *P. coronata* sequences (45).

Sequences were aligned using Clustal X2 (30), with gaps and ambiguous regions adjusted manually and reintroduced to the data set as single-state characters where positional homology could be assessed.

A distance matrix was created using PAUP 4.0b10 (42) to identify duplicate sequences, with one representative of each group

retained for phylogenetic analysis. Phylogenetic analysis was performed using BEAST v1.4.8 (20) with a GTR + I + γ model, empirical base frequencies, four gamma categories, and a relaxed lognormal molecular clock. The Yule process speciation prior was set as the means of speciation. For the analyses to reach convergence, the complete dataset was run for 100,000,000 generations and the *P. coronata* analysis for 10,000,000 generations. Burn-in was evaluated using Tracer v1.4 (<http://beast.bio.ed.ac.uk/Tracer>), with the first 10,000 trees discarded from the complete rust fungus analysis and the first 1,000 trees from the *P. coronata* analysis. TreeAnnotator v1.4.8 (<http://beast.bio.ed.ac.uk/TreeAnnotator>) was used to select for the maximum clade credibility tree for each analysis. Posterior probability summaries were calculated for all nodes that had a posterior probability greater than 0.5. Finished tree files were visualized in FigTree v1.2.3 (<http://tree.bio.ed.ac.uk/software/figtree/>).

Real-time PCR for species identification. Real-time PCR hydrolysis probes were designed for each of the three rust fungal species identified in this study. A complete list of new primers and probes developed can be found in Table 2. Probes PuSTM-ITS1 for *P. graminis* and PuSTR-ITS1 for *P. striiformis* were designed in the ITS1 region and were optimized for use with forward and reverse primers FrITS1Pu and RrITS1Pu. A *P. coronata* probe, PuCR-ITS2, was designed in the ITS2 region in combination with primers FrITS2Cr and RrITS2Cr. PuSTR-ITS1 was labeled on the 5′ end with the fluorescent reporter dye 6-carboxy-fluorescein (FAM) and on the 3′ end with the fluorescent quencher dye Black Hole Quencher 1 (BHQ-1) (IDT, Coralville, IA). PuSTM-ITS1 was labeled with the 5′ fluorescent reporter dye cyanine 3 (Cy3) and the 3′ quencher BHQ-2 (IDT), allowing for multiplexing with PuSTR-ITS1. PuCR-ITS2 was labeled on the 5′ end with the fluorescent reporter dye FAM and on the 3′ end with the fluorescent quencher dye Iowa Black Fluorescent Quencher (IBFQ) (IDT). An additional internal quencher, ZEN (IDT), was positioned in the center of the PuCR-ITS2 probe when this modification became available from the manufacturer to enhance specificity. Two additional primer/probe sets, PgFAM1 and PsFAM2, previously designed for detection of *P. graminis* f. sp. *tritici* and *P. striiformis* from cereal crops (8), respectively, were synthesized for comparison with the turfgrass rust primers and probes developed in this study. Both primer/probe sets also detected select sequences in the ITS1 region and were labeled on the 5′ end with the fluorescent reporter dye FAM and the quencher BHQ-1 on the 3′ end.

Positive controls were generated by synthesizing long oligonucleotides corresponding with the 218- or 219-bp or 198-bp region amplified in the real-time assays for each fungal species in the pUC57 plasmid vector (GeneWiz, Inc.). By synthesizing each species’ ITS amplicon in their own pUC57 plasmid vector, we ensured that positive controls did not contain mixed DNA concentrations of the three rust fungal species, as may occur when using samples collected in the field as positive controls. To avoid overloading the reaction with plasmid template DNA, positive-control plasmid DNA at 15 ng/μl was mixed with an equal part of sheared salmon sperm DNA (Invitrogen, Carlsbad,

Table 2. Polymerase chain reaction primers and hydrolysis probes developed in this study

Primer/probe ^a	Description	Sequence (5′–3′) ^b	Location (bp)
FrITS2Cr	<i>Puccinia coronata</i> forward ITS2	TTTGTGGATGTTGAGTGTTC	465–485 ^c
RrITS2Cr	<i>P. coronata</i> reverse ITS2	TCCACCTGATTTGAGGTCT	644–663 ^c
FrITS1Pu	<i>P. graminis</i> and <i>P. striiformis</i> forward ITS1	CCTGCGGAAGGATCATTATT	37–56 ^d
RrITS1Pu	<i>P. graminis</i> and <i>P. striiformis</i> reverse ITS1	TTTGGTTACATTCATTAAACTTGTG	230–255 ^d
PuCR-ITS2	<i>Puccinia coronata</i> probe	FAM-TACTTGCCA-ZEN-TCTTTTGAAAGGAGGGA-IBFQ	587–612 ^c
PuSTM-ITS1	<i>Puccinia graminis</i> probe	Cy3-TTAGAGTGCACTTTATTGTGGCTCAACTCTCT-BHQ2	64–95 ^d
PuSTR-ITS1	<i>Puccinia striiformis</i> probe	FAM-CGTAACTTCTTTATTGAATGTTGCATTACCCTCCC-BHQ1	160–194 ^d

^a ITS = internal transcribed spacer.

^b FAM = 6-carboxy-fluorescein fluorescent reporter dye, ZEN = internal quencher to enhance specificity, IBFQ = Iowa Black Fluorescent Quencher, Cy3 = cyanine 3 fluorescent reporter dye, BHQ-2 = Black Hole Quencher 2, and BHQ-1 = Black Hole Quencher 1 (IDT, Coralville, IA).

^c Primer/probe set base pair position in ITS2.

^d Primer/probe set base pair position in ITS1.

CA). A previously designed real-time PCR primer/probe set, StdLSU1, specific to the fungal large subunit region, served as an internal standard control (8).

Real-time PCR reactions were performed using a Cepheid SmartCycler (Cepheid, Sunnyvale, CA) in 25- μ l Cepheid tubes. Cepheid's Smartmix HM lyophilized PCR master mix was used for all optimization reactions, and Roche's Light Cycler 480 Probes master mix (Indianapolis, IN) was used for 96-well plate assays on the Step One Plus system (Applied Biosystems). Optimal cycling conditions were determined by using concentration gradients of 10 to 1 μ M of primers and probes at different annealing temperatures (54 to 60°C), and the cycle with the earliest detection was retained for all further analysis. The final reaction volumes contained 5 μ l of DNA and 20 μ l of Cepheid master mix, with 10 μ M both primers and 1 μ M probe. Final cycling conditions were as follows: initial denaturation at 95°C for 120 s, followed by 45 cycles of 95°C for 3 s and 60°C for 39 s. Manual threshold fluorescent units were set to 30 for *P. graminis* and 5 for *P. striiformis* and *P. coronata*.

Each probe was evaluated for specificity by testing all samples against each probe in a 96-well format, with each run replicated three times (Supplementary Table 1). Each sample was also screened using probes Pg FAM-1 and Ps FAM-2 (8), designed for detection of *P. graminis* f. sp. *tritici* and *P. striiformis* in wheat. Probes were considered specific if cycle threshold (C_T) values were zero for non-target DNA and water controls.

Assay sensitivity was determined by generating 10-fold dilutions of genomic DNA until target DNA could not be detected, with concentrations between 3 μ g/ μ l and 40 ng/ μ l. Assays were further examined by preparing reactions containing DNA extracted from 50, 100, 150, 200, and 300 individual urediniospores for each of the species to determine lower detection limits. Standard curves were generated using the SmartCycler (Cepheid) software to estimate quantities of DNA in unknown samples and to calculate reaction efficiency.

Results

Phylogenetic identification of turf rust species based on the ITS gene tree. In fall 2008, 66 turfgrass samples exhibiting symptoms of rust disease were collected from 13 U.S. states and four countries (Table 1). Phylogenetic analysis of the ITS-5.8S rDNA region identified *P. coronata*, *P. graminis*, and *P. striiformis* (Fig. 1) from infected leaf tissue. Based on their placement in the phylogeny in monophyletic groups along with the ITS sequences from exemplar isolates of cereal rust fungi, 67% of the turf samples were identified as *P. coronata*, 28% as *P. graminis*, and 5% as *P. striiformis* (Table 1; Figs. 1 and 2). *P. coronata* was found on seven different grass hosts: bromegrass (*Bromus* sp.), tufted hair grass (*Deschampsia* sp.), Kentucky bluegrass, perennial ryegrass, reedgrass (*Calamagrostis* sp.), tall fescue (*Festuca arundinacea* Schreb.), and zoysia grass (*Zoysia* sp.) (Table 1). In contrast, *P. graminis* was identified on a much smaller host range, consisting

Fig. 1. Maximum clade credibility Bayesian phylogenetic tree generated from sequences of the internal transcribed spacer-5.8S rDNA region of the three *Puccinia* spp. identified on turfgrass hosts in this study: *Puccinia coronata*, *P. graminis*, and *P. striiformis*. The *P. coronata* complex is expanded in Figure 2. Posterior probabilities >50% supporting the topology are represented by thick black lines and numerical values. Symbols next to taxa indicate host plant origin. Branch lengths are proportional to levels of sequence divergence. The tree is rooted using the outgroup taxa *P. hordei* and *P. recondita*.

of tall fescue and Kentucky bluegrass. *P. striiformis* was identified on only two grass hosts, perennial ryegrass and annual bluegrass (*Poa annua* L.).

All three rust fungal species formed well-supported monophyletic groups, with posterior probabilities ≥ 0.99 (Fig. 1). Within the three species clades, the turfgrass isolates clustered together and were separate from isolates from non-turf gramineous hosts (posterior probabilities ≥ 0.91). Within these divisions, there was no segregation based on geography, although sampling was not designed to test geographic distribution. *Puccinia striiformis* isolate AU-1, the only isolate in the study from Australia, diverged earlier in the phylogenetic tree than other members of the species but sampling was insufficient to determine whether this diversity reflected the unique geographic origin of this isolate relative to other isolates in the sample.

Several turfgrass samples within *P. coronata* and *P. graminis* formed well-supported subgroupings within their respective clades (Figs. 1 and 2); however, there were only four of these subgroups. Overall, outside of the division between cereal and

turf samples in each of the three species, little intraspecific variation was observed.

Real-time PCR identification of turfgrass rust species. The application of host plant association and spore phenotype characters incorrectly identified 42% of the rust fungus samples (Supplementary Table 2), with over half of these misidentifications coming from samples of rust fungi on Kentucky bluegrass. Using classical field identification techniques, 58% of the samples from Kentucky bluegrass would have been identified as *P. striiformis* using pustule phenotype or *P. graminis* using host plant association, instead of *P. coronata* as verified by ITS sequence data. Other misidentifications occurred where *P. coronata* and *P. striiformis* could not be accurately differentiated on hosts other than Kentucky bluegrass.

Using existing real-time PCR protocols developed for *P. graminis* f. sp. *tritici* and *P. striiformis* from wheat to discriminate between turfgrass samples in our study (8), results were variable. Data using the *P. graminis* probe Pg-FAM-1 were more reliable than data using the *P. striiformis* Ps-FAM-2 probe, which frequently produced false positives. However, neither probe

Fig. 2. Maximum clade credibility Bayesian phylogenetic tree generated from sequences of the internal transcribed spacer-5.8S rDNA region of the *Puccinia coronata* samples identified on turfgrass hosts in this study. Posterior probabilities $> 50\%$ supporting the topology are represented by thick black lines and numerical values. Symbols next to taxa indicate host plant origin. Branch lengths are proportional to levels of sequence divergence. The tree is rooted using the outgroup taxa *P. graminis*.

consistently identified the different turf rust fungal species correctly. The existing Pg-FAM-1 probe sequence contained one unique single-nucleotide polymorphism (SNP) not found in *P. graminis* turfgrass isolates, located at position 5 in the probe region. The Pg-FAM-1 probe sequence was also not found in *P. coronata* or *P. striiformis* samples from turfgrass. Ps-FAM-2 was identical in sequence to *P. striiformis* samples NYH-4 and PA-2; however, the sequence was not found in sample AU-1, thus limiting its application for the detection of all *P. striiformis* isolates from turf. Therefore, we used the ITS sequence from our phylogenetic analyses to develop a real-time PCR diagnostic assay specifically designed for identification of turfgrass associated rust fungus species.

Fluorescence-labeled hydrolysis probes and primer pairs for use in real-time PCR experiments were designed using the ITS sequence alignment as a template to identify variable sites between the three *Puccinia* spp. associated with turf-type gramineae hosts. Two probes, PuSTR-ITS1 and PuSTM-ITS1, were designed within the ITS1 region to detect *P. striiformis* and *P. graminis*. The probe region differed among the three species associated with turf-type gramineae hosts by at least 2 SNPs. The primer and probe binding sites for PuSTR-ITS1 and PuSTM-ITS1 in both turfgrass isolates and representative cereal isolates are illustrated in Figure 3. These probes were positioned within a 219-bp PCR amplicon that would be generated by the same primer pair for both species (Table 2).

Two attempts were made to also design a probe for *P. coronata* within the same ITS1 amplicon used for *P. graminis* and *P. striiformis* but neither of these probes produced acceptable levels of fluorescence or repeatable results (*data not shown*). Therefore, a probe for detection of *P. coronata*, PuCR-ITS2, was designed in

the ITS-2 region, positioned within a 199-bp PCR amplicon that contained a 6-bp deletion specific to turfgrass isolates of *P. coronata* (Fig. 4). When used to screen samples in the current study, real-time PCR analysis showed that each probe accurately identified samples from the target species. A summary of these data is found in Supplementary Figure 1A–C. Non-target DNA and negative controls produced C_T values = 0. Detection using PuSTR-ITS1 began at 50 urediniospores ($C_T = 32.74$), PuSTM-ITS1 at 150 spores ($C_T = 36.62$), and PuCR-ITS2 at 150 urediniospores ($C_T = 34.42$). Assay sensitivity determined by serial dilutions of genomic DNA showed the lowest levels of detection to be between 1 and 9 pg (Fig. 5). Positive controls that were synthesized in the pUC57 plasmid vector repeatedly produced fluorescence early in the PCR cycle, with C_T values of 20 to 25. Biological samples generated fluorescence curves later in the cycle, with C_T values of 27 to 38, and increased with decreasing DNA concentrations. Nonspecific positive reactions were observed from four targets (OH-1A/B and OH-8A/B); however, additional DNA sequencing of cloned ITS amplicons established the presence of both *P. coronata* and *P. graminis* in these samples.

Probes PuSTR-ITS 1 and PuSTM-ITS 1 were designed within the same primer set to allow multiplexing of the two probes with one another for simultaneous detection of *P. graminis* and *P. striiformis*. When screened against *P. graminis* and *P. striiformis* synthesized positive controls, the multiplex reaction successfully detected both positive controls at a C_T value ($C_T = 19.73$ and 18.11, respectively) comparable with non-multiplexed reactions (Fig. 6). The multiplexed reaction was also successful at detecting rust species from turfgrass samples collected in the field that were suspected of having rust infection. Detection was between C_T values of 21.60 and 23.97 (Fig. 6).

Fig. 3. Alignment diagram of the partial rDNA internal transcribed spacer 1 (ITS1) region showing the placement of polymerase chain reaction primers FrITS1Pu and RrITS1Pu with *Puccinia graminis* probe PuSTM-ITS1 and *P. striiformis* probe PuSTR-ITS1. Representative samples from *P. coronata*, *P. graminis*, and *P. striiformis* on turfgrass and cereal crop hosts are included in the alignment. Species-specific single-nucleotide polymorphisms (SNPs) differentiating species are denoted by gray and white shading. Probe binding sites differed between the three turfgrass rust fungi by at least 2 SNPs. Numbers next to species names represent nucleotide base pair positions.

Discussion

The primary objective of this study was to evaluate fungal species responsible for rust disease of turfgrasses using molecular phylogenetic analysis. From 66 diseased grass samples, three rust fungi were identified: *P. coronata*, *P. graminis*, and *P. striiformis*. All three of these fungi have been previously identified through spore morphology as causing rust on turfgrass hosts (18); however, this research is the first evaluation of turf rust fungi using molecular data. Two other species described as causing turfgrass rust diseases in temperate regions, *P. brachypodii* and *P. recondita*, were not identified in the current study with either morphological or phylogenetic analysis.

From an evolutionary perspective, all three species formed distinct monophyletic groups and were subdivided broadly according to host plant origin, in agreement with the findings of Zambino and Szabo (45). Our data showed no pattern of segregation based on locale in any of the clades, suggesting that turfgrass populations of these fungi are widespread and highly mobile, as is known to occur in the wheat stem rust pathosystem (29). Population-scale investigations are needed to test this idea further, because the sampling and molecular markers used in the present research were not designed to address distribution patterns.

In our analyses, subspecific groups corresponding to a split between *P. graminis* f. sp. *tritici* and *P. graminis* sampled from all

other hosts were recovered, an organization that has also been observed in previous research using ITS sequence data (1). Differentiation of the ITS sequences between these subspecific groups was well-supported by posterior probabilities, with 20 SNPs and 6 insertion/deletion events in the IT1 and ITS2 regions supporting these groups. In studies of *P. graminis* using an ITS gene tree, Abbasi and colleagues (1) put forth the idea that these subspecific groups might be recategorized as two distinct species but, to date, this possibility has not been robustly tested through comprehensive sampling of the fungus and multilocus phylogenetic studies capable of systematically identifying species boundaries.

Similarly, the *P. striiformis* clade was subdivided into two major groups: a perennial ryegrass group and a wheat/barley group. The subdivision of *P. striiformis* into separate species has yet to be adopted by many researchers, and standard forma speciales nomenclature continues to be applied (6,15,21,24,25). The recent description of four strongly supported monophyletic lineages within *P. striiformis* supports division of the species to *P. striiformis* sensu stricto on members of *Triticeae*, *P. pseudostriformis* on bluegrass, *P. striiformoides* on orchard grasses (*Dactylis* sp.), and *P. gansensis* on needlegrass (*Achnatherum* sp.) (2,32). Though sampling within the family *Poaceae* in this study was limited to perennial ryegrass and annual bluegrass, we found strong posterior probability support (>0.63) for subdivisions of *P. striiformis* on members of *Triticeae*, orchard grass, and cultivated turfgrass hosts,

Fig. 4. Alignment diagram of the rDNA internal transcribed spacer 2 (ITS2) region showing the placement of polymerase chain reaction primers FrITS2Cr and RrITS2Cr with *Puccinia coronata* probe PuCr-ITS2. Representative samples from *P. coronata*, *P. graminis*, and *P. striiformis* on turfgrass and cereal crop hosts are included in the alignment. Species-specific single-nucleotide polymorphisms (SNPs) differentiating species are denoted by gray and white shading. Probe binding sites differed between the three turfgrass rust fungi by at least 5 SNPs. Numbers next to species names represent nucleotide base pair positions.

consistent with the groupings by Liu and Hambleton (32). Although this suggests that *P. striiformis* isolates from perennial ryegrass may, in fact, be properly placed within *P. pseudostriiformis*, more robust sampling is needed to confirm the exact taxonomic position of perennial ryegrass isolates relative to those from bluegrass hosts. With the recent identification of barberry (*Berberis* spp.) as the alternate host of *P. striiformis* f. sp. *tritici* (syn. *P. striiformis* sensu stricto) and *P. striiformis* f. sp. *poae* (syn. *P. pseudostriiformis*) (27), it is likely that future research will serve to more

clearly define the relationships and boundaries that exist between *P. striiformis* sensu lato pathotypes, genetic populations, and differentiated species, as well as the methodologies used to discriminate between these entities.

Within the *P. coronata* phylogeny, two major subdivisions were observed: a group comprising samples from non-turfgrass hosts and a second group largely composed of rust fungal samples from turfgrass hosts (Fig. 2). In the turfgrass clade, additional subspecific clustering was also observed but these groups were not sup-

Fig. 5. Real-time polymerase chain reaction data with 10-fold dilutions showing decreased fluorescence as sample concentration decreases (left) and standard curve showing the straight line relationship between the log of known DNA concentrations and the second derivative threshold cycle value (right). ITS = internal transcribed spacer. **A** and **B**, *Puccinia coronata* probe PuCr-ITS2 with lower detection limit of 9 pg; **C** and **D**, *P. graminis* probe PuSTM-ITS1 with lower detection limit of 1 pg; and **E** and **F**, *P. striiformis* probe PuSTR-ITS1 with lowest detection limit of 4 pg.

Fig. 6. Real-time polymerase chain reaction data with *Puccinia graminis* probe PuSTM-ITS1 and *P. striiformis* probe PuSTR-ITS1 multiplexed in a single reaction tube using turfgrass samples. ITS = internal transcribed spacer. Only target of interest produced fluorescence in each multiplexed reaction. Positive control pUC57 samples are labeled. **A**, Fluorescence for the 6-carboxy-fluorescein-labeled probe PuSTR-ITS1 and **B**, fluorescence for the cyanine 3-labeled probe PuSTM-ITS1.

ported through posterior probability estimates. In these subspecific clades, there was almost no supported resolution between samples, reflecting the high levels of sequence similarity. This similarity was confirmed when pairwise distances were calculated for the isolates. Intraspecific distances ranged between 0.00168 and 0.10774, with 46% of the ITS sequences identical ($P = 0.000001$) to at least one isolate from the sample (*data not shown*).

Real-time PCR for the accurate and quick identification of turfgrass rust species. Identification of *P. graminis* infecting turfgrass samples through the use of spore color phenotype has been long considered a reliable process, because the pustules exhibit an easily distinguishable color that has been thought to separate the fungus from *P. coronata* and *P. striiformis* (40). Phenotypic identification of *P. graminis* from turf has also been used in conjunction with host association criteria, because the fungus was expected to be found in association with bluegrass but not ryegrass hosts (40,43). Inaccurate identification of turfgrass rust fungi has been most commonly associated with the differentiation between *P. coronata* and *P. striiformis*, because the urediniospores exhibit a similar yellow-orange color and the characteristic linear nature of *P. striiformis* uredinial pustules can be difficult to distinguish on narrow leaf blades (32).

Real-time PCR has been previously shown to be effective for discrimination and detection of several rust fungi, including *Phakopsora pachyrhizi*, *Puccinia graminis* f. sp. *tritici*, *P. kuehnii*, *P. melanocephala*, *P. polysora*, *P. recondita*, *P. sorghi*, *P. striiformis* (from cereals), and *P. tritricina* (8,9,17,23,27). Although the ITS sequence data were not capable of identifying subspecific groups with any degree of confidence in our analysis, we were able to demonstrate the ability of the ITS region to accurately identify turfgrass rust fungi, and used the sequence data as the basis for the development of a real-time PCR protocol. Even minute amounts of DNA can be detected through this protocol (as little as 1 to 9 pg or 50 to 150 urediniospores), with an accurate identification provided from DNA samples in only 30 min. The assay was 97% reliable in its identification of *P. graminis*, *P. coronata*, and *P. striiformis* from turfgrass tissue; could detect the presence of multiple species in mixed infections; and, once implemented in the laboratory, can be routinely performed by turfgrass breeding programs, plant health practitioners, and diagnostic clinics with minimal training in molecular techniques. Already, the Plant Diagnostic Laboratory at Rutgers University is using the real-time PCR probes to identify turfgrass rust fungal species in support of breeding efforts at the University (R. Buckley and S. Tirpak, *personal communication*). The use of synthesized positive control DNA and multiplexing probes significantly reduces the number of steps needed to successfully implement this procedure and eliminates the need for the collection and validation of control samples of the three turfgrass rust fungi. This assay provides a way to rapidly and accurately identify turfgrass rust fungi and, thus, should become a valuable tool for diagnosticians as well as turfgrass managers seeking to control rust diseases in the future.

***P. coronata* as a widespread pathogen of Kentucky bluegrass.** Traditionally, turfgrass breeders have used pustule phenotype and host plant identity to discriminate between rust pathogens in the field. Using this system, a red pustule phenotype would indicate stem rust and an orange phenotype crown or stripe rust. Crown and stripe rust have been further differentiated by the orientation of pustules along the leaf vein, with stripe rust pustules forming parallel to the host vein (40), although recent research has shown that orientation of the sorus is an unreliable feature (32). Using these criteria, stem rust caused by *P. graminis* has been the most frequently reported rust disease on Kentucky bluegrass, while crown rust caused by *P. coronata* has been the most common disease on ryegrass hosts (40,43). However, it is now apparent that traditional phenotype and host association criteria are imprecise methods for the identification of fungi causing rust in turfgrass hosts, because our data showed *P. coronata* to be the most common rust pathogen on Kentucky bluegrass, and both *P. graminis* and *P. striiformis* were found in association with both rye and bluegrass

hosts. Consequently, the breakdown of resistance to rust that was observed in Kentucky bluegrass during the past decade may not have occurred due to the emergence of new *P. graminis* races as originally hypothesized (10), a finding that is consistent with the long-term, stable race structure of *P. graminis* in the United States since the eradication of barberry across the Great Plains. Instead, our data support an alternative scenario: that the upsurge of rust disease in Kentucky bluegrass could be the result of an increased association of this grass with *P. coronata*. Kentucky bluegrass is a documented host of *P. coronata* in New Zealand, the United Kingdom, Poland, China, and the Czech Republic; however, this is the first published report of this host/fungus association from the western hemisphere (22). Nevertheless, *P. coronata* has occurred on Kentucky bluegrass in North America in the past, because our observations of herbarium specimens revealed the presence of the fungus at least as far back as the 19th century (e.g., herbarium specimen BPI060420 in the U.S. National Fungal Collections from Missouri, collected 30 October 1886). Whether this “novel” host-pathogen association is truly new or is a longstanding relationship that has gone unnoticed due to misidentification has yet to be resolved. Alternatively, the possibility that the fungus recently transitioned from another host population to Kentucky bluegrass cannot be ruled out and is, in fact, supported by the clustering of *P. coronata* isolates from Kentucky bluegrass, perennial ryegrass, and tall fescue with the pathogen from oat (*Avena sativa* L.; Fig. 2). Because *P. coronata* is highly variable (18), reports of the fungus causing new diseases in economically important grasses are not uncommon, as evidenced by its identification as a new pathogen of barley (*Hordeum vulgare* L.) (26) and smooth brome (*Bromus inermis* Leyss.) (19).

The identification of *P. coronata* as a common, widespread pathogen of Kentucky bluegrass is likely to have considerable impact on future turfgrass breeding efforts. Unlike with the *P. graminis* and *P. striiformis* pathosystems, the alternate host for *P. coronata*, common buckthorn (*Rhamnus cathartica* L.), is an invasive and widely distributed plant in North America, allowing *P. coronata* to complete its sexual cycle. As a result, in cereal pathosystems afflicted by *P. coronata*, new virulent races are continually evolving through genetic variation generated by sexual recombination and the rapid appearance of random mutations. For example, between 2001 and 2005, 383 unique races of *P. coronata* were documented from 680 oat samples in the United States, with mean virulence significantly increasing during this period (14). Such a scenario in Kentucky bluegrass could have far-reaching implications for the turfgrass industry but remains to be validated.

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