MOLECULAR ANALYSIS AND INFECTIVITY OF *PUCCINIA* SPECIES
PATHOGENIC TO TURFGRASS

by

LISA A. BEIRN

A thesis submitted to the
Graduate School-New Brunswick
Rutgers, The State University of New Jersey
in partial fulfillment of the requirements
for the degree of
Master of Science
Graduate Program in Plant Biology
written under the direction of
Dr. Bruce B. Clarke and Dr. Jo Anne Crouch
and approved by

________________________
________________________
________________________

New Brunswick, New Jersey
May, 2011
ABSTRACT OF THE THESIS

Molecular Analysis and Infectivity of *Puccinia* Species Pathogenic to Turfgrass

By LISA A. BEIRN

Thesis Directors:

Dr. Bruce B. Clarke and Dr. Jo Anne Crouch

Rust (*Puccinia* spp.) has become an increasingly prevalent disease of cool-season turfgrasses over the past two decades. Unlike the cereal rust pathosystem, pathogen biology, populations, races, and species of rust fungi associated with turfgrasses have not been well studied. In this thesis, isolates of turfgrass rust fungi from the United States and four other countries were evaluated for genetic variation within the ribosomal internal transcribed spacer (ITS) region and were identified using phylogenetic analysis. Three rust species were revealed in the sample collection: *Puccinia coronata*, *P. graminis*, and *P. striiformis*. *P. coronata* was frequently found in association with Kentucky bluegrass (*Poa pratensis*), a host/pathogen relationship that has not been previously reported. Since the ITS region exhibited sufficient nucleotide variation for the identification of turfgrass rust species within the sample collection, this region was used to design species-specific primers and probes for the development of an accurate, reliable, real-time PCR assay to rapidly diagnose rust fungi from diseased turfgrass tissue. Accurate, reproducible species identifications were made using as few as 50-150 urediniospores, even in mixed infections. This protocol was designed so that diagnosticians, pathologists, and turfgrass breeders who may have limited molecular
training can identify rust species on turfgrass with relative ease. 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 error-prone field-based identification techniques. However, research and breeding efforts are still impeded by the inability to rapidly screen new turfgrass germplasm for resistance to rust and difficulty in obtaining pure isolates of rust fungi for genotyping analysis. By adapting and modifying a protocol designed for cereal rust fungi, an inoculation procedure was developed for two turfgrass rust species prevalent in temperate regions: *P. coronata* and *P. graminis*. Uredinia of both species were induced on Kentucky bluegrass cultivars Bewitched, Diva, and Ulysses 14 days after inoculation. The molecular, diagnostic, and infectivity studies described in this thesis will serve as a foundation for future research on the rust fungi that attack turfgrass.
ACKNOWLEDGMENTS

I would like to thank my advisors Dr. Bruce Clarke and Dr. Jo Anne Crouch for their guidance and assistance over the past two years. Their vast expertise has been invaluable to both my research and personal development and I am grateful to have had the opportunity to work with them. I would also like to thank Dr. William Meyer for serving on my committee, guiding and assisting me in field collections, and for the many helpful discussions.

I would also like to express gratitude to Dr. Bradley Hillman, Dr. Donald Kobayashi, and Dr. Melinda Moy, who graciously contributed their time, materials, and intellect to assist my research. I am also thankful to Dr. Nrupali Patel, for her support as both a mentor and friend. In addition, I would like to acknowledge Mark Peacos for helping maintain my greenhouse studies, as well as his assistance in fixing, designing and constructing research equipment.

I am also appreciative to the Rutgers Center for Turfgrass Science, whose generous financial assistance has helped fund my graduate assistantship and support this research.

Finally, I would like to thank my family and friends for their unconditional love and guidance. Their continued support and encouragement has helped me achieve my aspirations and become the strong, independent person that I am. I am forever thankful to all of them and am extremely grateful to have them in my life.

Chapter 1 has been submitted to the peer-reviewed journal *Plant Disease*. It is currently under review (PDIS-01-11-0073-RE).
TABLE OF CONTENTS

ABSTRACT OF THE THESIS............................................................................................................ii
ACKNOWLEDGMENTS....................................................................................................................iv
TABLE OF CONTENTS..................................................................................................................v
LIST OF TABLES..........................................................................................................................vii
LIST OF FIGURES........................................................................................................................ix
LITERATURE REVIEW....................................................................................................................1
  Rust Fungi: A Brief Introduction.................................................................................................1
  Rust Diseases of Cereals and Grasses.........................................................................................2
  Turfgrass Hosts..........................................................................................................................5
  Pathogen Biology: Taxonomy and Classification.........................................................................6
  Lifecycle......................................................................................................................................8
  Disease Cycle and Epidemiology...............................................................................................11
  Control.......................................................................................................................................13
  Rust Disease Epidemics in Turfgrass.........................................................................................14
  References.................................................................................................................................16

CHAPTER 1. Molecular Analysis of Turfgrass Rusts Reveals the Widespread
  Distribution of Puccinia coronata as a Pathogen of Kentucky Bluegrass in the
  U.S. ..............................................................................................................................................21
  Abstract .......................................................................................................................................21
  Introduction..................................................................................................................................22
CHAPTER 2. Development of a Greenhouse-Based Inoculation Protocol for Isolates of
Puccinia coronata and P. graminis Pathogenic to Kentucky Bluegrass ........... 64

Abstract .............................................................................................................. 64
Introduction .......................................................................................................... 66
Materials and Methods ......................................................................................... 69
Results .................................................................................................................... 73
Discussion .............................................................................................................. 75
References .............................................................................................................. 78
Tables ....................................................................................................................... 80
Figures .................................................................................................................... 83
CURRICULUM VITAE ......................................................................................... 88
LIST OF TABLES

CHAPTER 1:

Table 1. Sources of rust fungal samples used in this study.................................47
Table 2. PCR primers and hydrolysis probes developed in this study.....................50
Table 3. Results of real-time PCR experiments using probes to screen fungal samples........................................................................................................51
Table 4. Comparison of turfgrass rust species identification using morphology, phenotype, host plant association, and sequence data techniques............54
CHAPTER 2:

Table 1. Fungal isolates used for the greenhouse-based inoculation protocol.................................................................................................................................................. 80

Table 2. Results of inoculation experiments using different Kentucky bluegrass cultivars and three Puccinia isolates................................................................. 81

Table 3. Morphological and real-time PCR evaluations of harvested Puccinia coronata and P. graminis urediniospores from three inoculated Kentucky bluegrass cultivars ........................................................................................................................................ 82
LIST OF FIGURES

CHAPTER 1:

Figure 1. Map depicting rust sampling locations in the United States....................56

Figure 2. Maximum clade credibility Bayesian phylogenetic tree of the three
Puccinia sp. identified on turfgrass hosts in this study.................................57

Figure 3. Maximum clade credibility Bayesian phylogenetic tree of P. coronata
complex identified on turfgrass hosts in this study.....................................58

Figure 4. Alignment diagram of the partial ITS 1 region showing the placement of
real-time primers FrITS1Pu and RrITS1Pu with P. graminis probe
PuSTM-ITS1 and P. striiformis probe PuSTR-ITS1.................................59

Figure 5. Alignment diagram of the ITS 2 region showing the placement of real-
time primers FrITS2Cr and RrITS2Cr with P. coronata probe PuCr-
ITS2........................................................................................................60

Figure 6. Real-time polymerase chain reaction data with 10-fold dilutions showing
the decreased fluorescence as sample concentration decreases and
standard curve showing the line relationship between the log of known
DNA concentrations and the second derivative Ct value.........................61

Figure 7. Real-time polymerase chain reaction data with P. graminis probe
PuSTM-ITS1 and P. striiformis probe PuSTR-ITS1 multiplexed in a
single reaction tube using turfgrass samples..........................................62

Figure 8. Real-time PCR amplification plot of turfgrass rust probes against a 96-
well plate of turfgrass rust isolates.........................................................63
CHAPTER 2:

Figure 1. Photograph depicting the plexi-glass misting chamber used to incubate Kentucky bluegrass plants previously inoculated with *Puccinia* species.................................................................83

Figure 2. Photograph depicting the custom-built wood framed inoculation chambers.....................................................................................................................84

Figure 3. *P. coronata* images from inoculated Kentucky bluegrass .................85

Figure 4. *P. graminis* images from inoculated Kentucky bluegrass.................86

Figure 5. Real-time PCR data from inoculation experiments.............................87
LITERATURE REVIEW

Rust Fungi: A Brief Introduction

The rust fungi are a unique group of obligate parasites that belong to the order Pucciniales of the class Basidiomycetes. It has been estimated that there are more than 7000 species in the group, of which only 3/4 are fully described (Hawksworth et al., 1995). The largest genus, *Puccinia*, contains about 4000 species (Abbasi, 1996), with approximately 650 of these species pathogenic to hosts within the family Poaceae (Abbasi, 1996). As their name implies, rust fungi form orange to reddish-brown colored spore bearing pustules (uredinia) on the surface of the host plant which makes these pathogens easily recognizable in the field. Unlike some fungal plant pathogens that require stressed or damaged hosts for infection, rust fungi colonize actively growing, succulent plant tissue (Cummins and Hiratsuka, 2003). They can attack a wide range of host plants, including; mosses, ferns, conifers, monocots, and dicots (Cummins and Hiratsuka, 2003). As a group, rust fungi can cause extensive damage to monocultures of agriculturally and economically important crops such as cereal and grasses (family Poaceae), flax (*Linum* spp. L.), coffee (*Coffea* spp. L.), soybeans (*Glycine max* (L.) Merr], sugarcane (*Saccharum officinarum* L.), cotton (*Gossypium* spp. L.), onions (*Allium* spp. L.), white pine (*Pinus strobus* L.), spruce (*Picea* spp. A. Dietr.), and junipers (*Juniperus* spp. L.) (Cummins and Hiratsuka, 2003). In agricultural regions prone to rust disease, they are often the limiting factor for the successful cultivation of these crops (Cummins and Hiratsuka, 2003).
Rust Diseases of Cereals and Grasses

Reports of rust fungi attacking cereal crops have been described in the literature as early as 1000 B.C., where they are often referred to as causing a ‘yellowing’ of plant tissue (Chester, 1946). In biblical times, this ‘yellowing’ was often thought to be the result of punishment from God for the sins committed by man (Chester, 1946). These beliefs continued for centuries and sacrificial rust festivals, such as the Roman celebration Robigala (~700 B.C.), became increasingly popular.

It was not until 384-322 B.C. that historical references begin to consider rust disease as something other than a punishment from the God(s) (Chester, 1946). Works by Aristotle and Theophrastus describe temporal conditions where warmth and excessive moisture appeared to result in severe rust epidemics (Chester, 1946). In the mid-1700's, Fontana became the first to recognize that rust disease of cereals was in fact caused by a fungus, and successfully published the first figures on the disease (Fontana, 1767). Since Fontana’s early work with rusts, cereal rust epidemics have continued to be noted throughout the 20th century in both developed and underdeveloped nations worldwide (Leonard and Szabo, 2005).

Rust fungi have also infected other grass hosts for centuries; however, these pathosystems have often been overlooked because of their relative unimportance in the global food arena. Grasses such as Kentucky bluegrass (Poa pratensis L.), tall fescue (Festuca arundinacea Schreb.), and perennial ryegrass (Lolium perenne L.) can also be severely affected by rust diseases (Smiley et al., 2005). Although not valued as food crops, these grasses are important for their economic and aesthetic value, both as pasture grasses and cultivated turfgrasses.
In a forage setting, tall fescue and perennial ryegrass are the predominate grass species (Kimbeng, 1999). When infected with rust, these grasses are often ignored by livestock, who typically prefer non-rusted grasses (Cruickshank, 1957). During a *Puccinia coronata* Corda (crown rust) infestation in New Zealand, only 18% of rust infected forage grasses were consumed by sheep, compared with 99% for the rust free plants (Cruickshank, 1957). Consuming leaf tissue with rust can reduce milk yields among Holstein-Friesian and Australian Friesian Sahiwal cattle (Lowe et al., 1985) and increase the susceptibility of livestock to disorders caused by *Fusarium* sp. (feed refusal, low weight gain) and *Pythomyces chartarum* Berkeley and Curtis (facial eczema) (Kimbeng, 1999). Because of the cost of controlling rust in pastures and the animal-human safety concerns associated with pesticide use, resistant cultivars are often the principal means of preventing rust diseases on forage grasses (Cruickshank, 1957).

Rusts can also cause significant damage in the turfgrass seed production industry. In 1994, tall fescue seed produced in Oregon was valued at $28 million (Miles, 1995), making it one of the most valuable crops in the Pacific Northwest. In a severe *P. coronata* epidemic of perennial ryegrass in the United Kingdom in 1987, seed yield was decreased by as much as 25% (Potter, 1987). In New Zealand, perennial ryegrass seed yield, dry matter, and tiller production has been reduced by 53% as a result of *P. coronata* infection (Lancashire and Latch, 1966). In the United States, a *Puccinia graminis* Pers.:Pers. (stem rust) epidemic can reduce perennial ryegrass seed yields by as much as 98% (Pfender, 2009b). Yield loss is also prevalent in tall fescue seed production fields in Oregon; where non-treated, rust infected plants can have lower dry weight and
reduced seed production, compared to rust free plants treated with propiconazole (Welty and Azevedo, 1996).

In contrast to pasture grasses, fungicide use is common in grass seed production. Two to 4 preventative applications of propiconazole are usually made throughout the season to perennial ryegrass and tall fescue seed production fields, at a cost of $40 or $50 per hectare (Pfender, 2001a). If conditions are particularly conducive to rust outbreaks, the overall cost can increase considerably. Due to the dramatic yield loss rust fungi can cause in grass seed production, and the expense associated with fungicide applications, disease severity models that estimate yield loss from rust are now used in the Pacific Northwest (Pfender, 2003).

Turfgrass is an important component of athletic fields, home lawns, parks, and commercial environments around the world. In the United States, turfgrass establishment and maintenance is one of the largest agricultural industries, contributing more than 40 billion dollars to the national economy each year (The Turfgrass Industry- Present and Future, available from http://www.turfresearch.org/initiative.htm). Like the previously described agricultural systems, rust diseases can cause considerable damage to turfgrass. Diseased turf typically exhibits an unsightly orange to brown discoloration that can weaken plants and reduce the aesthetic value of athletic fields, golf courses, parks, home lawns, and sod farms. Over time, affected areas may drastically thin (Bushnell, 1984) and require renovation. Chemical control can be costly and is sometimes ineffective. Thus, resistant cultivars are an important means of preventing rust disease in turf.
Turfgrass Hosts

In the temperate United States, *P. coronata* (crown rust), *P. graminis* var *graminis* (stem rust), *Puccinia striiformis* Westend. (stripe rust), and *Puccinia recondita* Roberge ex Desmaz. and *Puccinia brachypodii* G. Otth, (leaf rusts), are considered the most prevalent rust pathogens of turfgrasses (Smiley et al., 2005). These fungi have long been considered host specific, with isolates of *P. graminis* causing disease on fescue, ryegrass, bluegrass, and Timothygrass (*Phleum* sp.), *P. striiformis* causing disease on wheatgrass (*Agropyron* sp.), bentgrass (*Agrostis* sp.), fescues, ryegrass, Timothygrass, and bluegrass, *P. coronata* causing disease on wheatgrass, bentgrass, fescues, ryegrass, paspalums (*Paspalum* sp.), and Timothygrass, and *P. recondita* and *P. brachypodii* causing disease on wheatgrass, bentgrass, fescues, ryegrass, and bluegrass (Smiley et al., 2005). In the southern United States, where warm-season turfgrasses predominate, multiple rust fungi are reported to infect bermudagrass (*Cynodon* sp.), paspalums, and zoysiagrass (*Zoysia* sp.), and these pathogens appear to be host specific (Smiley et al., 2005). Additionally, *Puccinia, Physopella*, and *Uromyces* species have been described from Asia, Europe, and South America, many of which are considered host specific on grasses only found in those regions (Smiley et al., 2005). However, caution must be used when considering the host range for graminicolous rust species, as cross-infectivity has been reported with isolates of *P. graminis* obtained from perennial ryegrass and tall fescue (Pfender, 2009a, 2001b).
Pathogen Biology: Taxonomy and Classification

Until recently, classification and taxonomic divisions of graminicolous rust fungi were based solely on morphological characteristics (Cummins, 1971). Because spore morphology is highly variable, it is not unusual for the number of taxonomically accepted species to differ between studies (Cummins and Hiratsuka, 2003). Presence or absence of paraphyses, ornamentation of the urediniospore, arrangement of germ pores, teliospore pedicel type, and number of cells in the teliospore are typically regarded as the most important spore traits for the identification of rust species (Cummins, 1971); however, several graminicolous rust species have been shown to exhibit intermediate teliospore forms (Maier et al., 2007), or have characteristics, such as teliospore pedicel type, that can vary between closely related groups (Maier et al., 2007), complicating accurate identification or classification. For example, *Puccinia ellisiana*, the causal agent of rust on bluestem grass (*Andropogon* sp.) is known to display phenotypic diversity when sporulating on different aecial hosts (Long, 1914). Currently, revisions are being made to the morphological identification techniques used within the Pucciniales, with more emphasis being placed on spermogonial structure, rather than teliospore morphology (Cummins and Hiratsuka, 2003).

At present, there are a total of 39 different species of rust fungi (*Puccinia*, *Physopella*, and *Uromyces*) known to be pathogenic to turfgrasses (Smiley et al., 2005). This differs from Cummins assessment in 1971, which used teliospore and urediniospore morphology to describe 29 *Puccinia* species, one *Physopella*, and four *Uromyces* species. More recently, 56 species of *Puccinia* and 14 *Uromyces* species have been associated with infestations of the family Poaceae which includes turf and non-turf hosts (Afshan,
Currently, graminicolous rust fungi are classified as follows: phylum Basidiomycota, subphylum Pucciniomycotina (= Urediniomycetes sensu Swann and Taylor 1995), class Pucciniomycetes, order Pucciniales (= Uredinales), and family Puccinaceae (Aime et al., 2006, Index Fungorum accessed 6 April 2010). Given the complications with rust taxonomy based on morphology (Long, 1914, Maier et al., 2007), the use of molecular sequence data, combined with morphology, has become a powerful tool for the study of these fungi at the species level (Aime et al., 2006, Maier et al., 2007, Van der Merwe et al. 2007).

Ribosomal DNA sequence data has established the polyphyletic nature of both the *Puccinia* and *Uromyces* (Maier et al. 2003), that is, *Puccinia* and *Uromyces* do not share a most recent common ancestor, but do share some similar traits, such as one-celled teliospores (Van der Merwer et al., 2007). This finding is further corroborated by studies using the translation elongation factor 1α (TEF1α) and beta-tubulin (β-tubulin) genes (Van der Merwe et al., 2007). Within these polyphyletic genera, two distinct lineages have emerged that reflect evolutionary adaptations (Maier et al., 2007). Lineage 1 includes rusts pathogenic to hosts in the Cyperaceae, whereas lineage 2 contains rusts pathogenic to hosts in the Poaceae; demonstrating the early adaptation of *Puccinia* rusts to wet areas, followed by adaptation to a much drier environment (Maier et al., 2007, Van der Merwe et al., 2007).

Additionally, the internal transcribed spacer (ITS) region has been proven effective at delineating relationships within the genus *Puccinia* (Abbasi et al., 2005, Liu and Hambleton, 2010, Szabo, 2006, Zambino and Szabo, 1993). Phylogenetic analysis of the three main rust species found in temperate regions- *P. coronata*, *P. graminis*, and
*P. striiformis*- have supported multiple subdivisions within these species (Abbasi, 2005, Liu and Hambleton, 2010, Szabo, 2006). Using ITS sequence data, Szabo (2006) found *P. coronata* isolates obtained from perennial ryegrass to group with those from oats (*Avena sativa* L.), while isolates from bromegrass (*Bromus* sp.) formed their own clade. Similar subdivisions have been reported within *P. graminis*, where isolates from Kentucky bluegrass clustered separately from isolates from wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) (Abbasi, 2005), supporting early work conducted by Zambino and Szabo (1993). Morphological and sequence data (ITS, β-tubulin) have supported four species within *P. striiformis*: *P. striiformis* sensu stricto on Triticeae, *P. pseudostriiformis* on bluegrass, *P. striiformoides* on orchard grasses (*Dactylis* sp.), and *P. gansensis* on needlegrass (*Achnatherum* sp.) (Liu and Hambleton, 2010). The *P. striiformis* lineages described by Liu and Hambleton (2010) are strongly corroborated by both morphological and sequence data, thus it is likely that similar techniques will be needed to properly confirm subdivisions proposed in other graminicolous rust species, such as *P. coronata* and *P. graminis*.

**Lifecycle**

Turfgrass rust fungi are unique in that they are heteroecious, completing their life cycle on two unrelated host plants, and macrocyclic, producing five different spore types (Smiley et al., 2005). In the spring, overwintering dikaryotic teliospores on the turfgrass host begin to germinate, undergo karyogomy and meiosis, and produce four haploid basidiospores (Agrios, 2005). The basidiospores are forcefully ejected and travel via wind currents to an alternate host, where they undergo sexual recombination. The
alternate host for *P. graminis* and *P. striiformis* is barberry (*Berberis* spp.) (Leppik, 1961, Jin et al., 2010), whereas the common buckthorn (*Rhamnus cathartica* L.) serves as the alternate host for *P. coronata* (Dietz, 1926). Inoculation studies have shown that other woody ornamentals such as the Alabama supplejack [*Berchemia scandens* (Hill) K. Koch] and the russet buffaloberry [*Shepherdia canadensis* (L.) Nutt.] are capable of serving as an alternate host for *P. coronata*, however, common buckthorn is regarded as the primary alternate host for crown rust (Dietz, 1926).

Following ejection, dissemination, and landing on a suitable alternate host, the basidiospore begins to germinate. Shortly after germination, the germ tube penetrates the host’s epidermis (Agrios, 2005). Mycelium begins to form intercellularly, and within 3 to 4 days, a spermagonium ruptures through the host surface (Agrios, 2005). Haploid spermatia are excreted in a sticky, sweet exudate that attracts insects who serve as a vector to carry spermatia to other nearby spermagonia (Cummins and Hiratsuka, 2003). When a spermatium comes in contact with a compatible spermagonium, fertilization takes place, resulting in the formation of dikaryotic cells (Agrios, 2005). These dikaryotic cells grow through the leaf toward the lower leaf surface, forming aecia (Agrios, 2005). Unicellular aeciospores are produced in aecia, which are released in late spring, and travel via wind currents to susceptible graminicolous hosts. Following germination, mycelia begin to proliferate just below the epidermis of the host to form a uredium (Agrios, 2005). As numerous urediniospores are produced within the developing uredium just beneath the epidermis, pressure continues to increase, until the pustule ruptures through the surface and urediniospores are released (Agrios, 2005).
Urediniospores function as repeating spores and are the most important spore stage for the development of rust on turfgrass hosts. These spores are responsible for the yellow-orange turf discoloration seen in the field and for continuing or initiating new epidemics kilometers away from their point of origin (Kolmer, 2005). It is also during this stage that the most severe damage occurs in graminicolous hosts. Plant respiration and transpiration are increased, photosynthesis is decreased, and nutrients are lost as a result of infection by the rust fungus, eventually resulting in plant death (Agrios, 2005). Just before the plants are about to die, or unfavorable weather conditions are encountered, the fungus begins to produce two-celled teliospores, which can then overwinter on decaying debris and initiate new epidemics the following year (Agrios, 2005).

The unique, macrocyclic, and obligate nature of graminicolous rusts significantly complicates the study of these organisms. In vitro culturing of grass rusts has been extremely difficult; reports in the literature state the process is laborious, inefficient, and frequently produces inconsistent results (Williams et al., 1967). Axenic cultures of rust fungi have been successful by using a mixture of Czapek’s minerals, sucrose, yeast extract, and peptone; though this process can take several weeks (Huang et al., 1990, Williams et al., 1966, 1967). At present, to avoid the inconsistencies associated with axenic culturing, the standard method for maintaining isolates of cereal and grass rust fungi is through the continued inoculation of susceptible hosts in the greenhouse.

Typically, urediniospores are sprayed in an oil suspension onto host plants and then incubated under conditions favorable for rust development (Pfender 2009a, 2001a,b). Upon the appearance of uredinia, a single pustule is selected and allowed to develop, ensuring pathogen homogeneity (Pfender 2009a, 2001a,b). Detached leaf assays have
also been developed (Jackson et al., 2008); however, the short longevity of the leaves makes this method problematic for the efficient production of urediniospores.

**Disease Cycle and Epidemiology**

In mild climates, previously infested turfgrass stands can serve as overwintering sites for rust fungi (Smiley et al., 2005). In more northern areas that experience moderate temperatures and adequate snow coverage, rust may also overwinter in debris or infected plants (Kolmer, 2005). In areas where overwintering does not occur, the urediniospores may be introduced to a susceptible area by means of wind dissemination; the light-weight nature of the urediniospores makes it possible for them to be carried for thousands of kilometers from their initial infection site (Kolmer, 2005). For example, *P. triticina* (wheat leaf rust) is known to migrate on southerly winds from overwintering sites in Mexico to wheat production areas in the Great Plains, Ohio Valley, the northeastern U.S., and Canadian prairies (Kolmer, 2005).

Once in contact with a susceptible stand of turfgrass, the infection process can begin. Generally, turfgrass stands that are subjected to stressful conditions such as drought, nitrogen deficiency, and low mowing height are much more susceptible to rust disease; however infections can occur in healthy turf (Smiley et al., 2005). Newly seeded areas are also more likely to become infected by rust fungi, though disease severity can vary with the season of planting (Pfender, 2004) and the cultivar. Perennial ryegrass planted in early fall (September) can exhibit severe *P. graminis* infection compared to fields seeded in late fall (November) (Pfender, 2004).
Following inoculation, rust fungi undergo a latent period that is regulated by ambient temperature (Pfender, 2001a). Typically, optimal conditions for development of *P. coronata* and *P. striiformis* infections include air temperatures between 10 to 20°C (Ash and Rees, 1994, Smiley et al., 2005), whereas *P. graminis* requires warmer temperatures (22 to 28°C) (Smiley et al., 2005, Pfender, 2001a). Both *P. coronata* and *P. striiformis* also prefer high light intensities for optimal infection (Ash and Rees, 1994, Smiley et al., 2005), whereas *P. graminis* requires a period of darkness, followed by high intensity light (Smiley et al., 2005).

Although light intensity and temperature are controlling factors in rust disease development, leaf surface moisture also plays an important role in disease development. While moisture is required for all *Puccinia* species to infect turf, at least two hours of leaf wetness are required for *P. striiformis* infection, with infection intensifying as the hours of leaf moisture increases (de Vallavieille-Pope et al., 1995). Together, these conditions favor uredinial development and the production of urediniospores. Uredinia are usually seen on the adaxial surface of the leaf blade, a result of differing leaf topography and fewer stomates on the abaxial surface (Roderick and Thomas, 1997).

When the turf foliage begins to dry, mature, or go dormant, teliospores are produced. The two-celled structures exhibit a dark brown/black pigment in mass and often go unnoticed, since they are not as obvious as the urediniospores (Smiley et al., 2005). Teliospores occur most frequently in taller, unmowed grasses and are often not the target of fungicide programs, due to their less visible nature. If left to overwinter, teliospores will germinate in the spring and produce basidiospores, thus completing the rust lifecycle (Smiley et al., 2005).
Control

Control of rust fungi in livestock pastures and turfgrass stands is primarily achieved through the use of resistant cultivars. While resistance to rust fungi in turfgrass hosts is poorly understood, traditional breeding techniques have been used to incorporate resistance into graminicolous hosts (Bonos et al., 2006). It is likely that rust resistance in turfgrass is governed by similar mechanisms as in cereal crops, where resistance is the result of a dominant gene in the host matching up with an avirulence gene in the pathogen (Barker et al., 2003, Braverman, 1966, Britton and Butler, 1965, Leonard and Szabo, 2005, Pfleger, 1973), but this can vary with different species/races of rust fungi. For example, four quantitative trait loci (QTL) markers have been associated with resistance to *P. coronata* in perennial ryegrass, of which two linkage groups have shown homology with chromosomes in oat that are known to confer resistance to *P. coronata* in that pathosystem (Muylle et al., 2005).

Demethylation inhibiting (DMI) fungicides are frequently used in seed production to control rust diseases, in addition to quinone outside inhibitor (QoI) products (Pfender, 2009b). However, the integration of resistance genes into commercial cultivars would not only be a more environmentally sound practice, but would also reduce costs associated with fungicide applications. The DMI and QoI fungicides are also labeled for rust control in turfgrass (Clarke et al., 2006); however, their use often requires repeated applications. In addition to chemical and genetic control options, sound management practices should also be implemented to reduce rust disease in turfgrass. To improve suppression of rust disease, turf should be maintained on a balanced fertilizer program, kept well irrigated, and mowed regularly (Smiley et al., 2005). Removing
infected clippings after mowing may reduce the inoculum potential and thus the number of new rust infections (Smiley et al., 2005). *Tuberculina* species have been known to parasitize rust species (Lutz et al., 2004); however, it remains to be determined if the introduction of a parasitic fungal species would result in adequate control of rust disease in the field.

**Rust Disease Epidemics in Turfgrass**

Breeding for rust resistance has been an important component of turfgrass breeding programs for a number of years. Through intraspecific hybridization and recurrent selection cycles, significant improvements have been made in producing cultivars of Kentucky bluegrass and perennial ryegrass with increased resistance to rust diseases, thus drastically reducing the need for fungicides (Bonos et al., 2006). However, within the past decade, there has been an unusual increase in turfgrass rust epidemics on Kentucky bluegrass in the mid-Atlantic and Northeastern U.S. (Bonos et al., 2006). Previously resistant cultivars, such as the Midnight types of Kentucky bluegrass, are now generally experiencing a greater incidence of rust disease, a scenario that has significant implications for turfgrass breeding programs, the seed production industry, and pasture settings. While breeding for new, resistant cultivars is ongoing, numerous field seasons are required to properly assess resistance to rust disease, a process that can be lengthy and laborious. As a result, pesticide and fertilizer inputs are likely to increase to control the upsurge in rust disease until new rust resistant cultivars can be released.

As with any plant disease, adequate disease management and control begins with a fundamental understanding of the pathogen. In the turfgrass rust system, our ability to implement effective control strategies and develop resistant cultivars has been hindered
by the lack of available information about the causal agents. Given the enormous economic and aesthetic problems associated with rust disease of turfgrass and the confusion in accurately identifying and classifying these pathogens, adequate control of rust disease is difficult. Therefore, improving turfgrass breeding programs and expanding knowledge of the turfgrass rust system is an important priority. Evaluating these fungi on a molecular level, developing an accurate identification procedure, and implementing an effective inoculation protocol to maintain pathogen cultures would improve our ability to study turfgrass rust fungi.
REFERENCES


CHAPTER 1. Molecular Analysis of Turfgrass Rusts Reveals the Widespread Distribution of *Puccinia coronata* as a Pathogen of Kentucky Bluegrass in the U.S.

**ABSTRACT**

Over the past ten 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 U.S. were evaluated using rDNA internal transcribed spacer region (ITS) sequence data. 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 non-molecular criteria. To avoid such misidentifications, a real-time PCR diagnostic protocol was developed for turfgrass-associated *P. graminis*, *P. coronata*, and *P. striiformis* using ITS sequence data. Accurate, reproducible, species-specific identifications were made using as few as 50-150 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 error-prone field-based identification techniques.
INTRODUCTION

Rust is a common fungal disease of cultivated turfgrass that can occur in all turf species and is found worldwide (Smiley et al., 2005). Multiple fungi are responsible for rust symptoms in turf. In temperate regions, the most prevalent turf rust fungi are *Puccinia graminis*, which causes stem rust disease, *Puccinia striiformis*, which causes stripe rust disease, *Puccinia coronata*, which causes crown rust disease, and *Puccinia recondita* and *Puccinia brachypodii*, which cause leaf rust diseases (Smiley et al., 2005). 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 (Bushnell, 1984). Although fungicides can be used to minimize disease by limiting urediniospore production, chemical control is costly and sometimes ineffective (Smiley et al., 2005). As a result, the use of resistant turf cultivars is an important mode of defense against rust pathogens.

Over the past ten years, turfgrass breeders have documented a gradual shift in susceptibility to rust among Kentucky bluegrass (*Poa pratensis*) cultivars, particularly the ‘Midnight’ types (Bonos et al., 2006). 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 for incorporating resistance in turfgrass hosts (Bonos et al., 2006). 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 (Barker et al., 2003, Braverman, 1966, Britton and Butler, 1965, Leonard and Szabo, 2005, Pfleger, 1973), although different genes may be required for specific fungal races of rust and other environmental or host factors may also be important (Roelfs, 1988). Breakdown of rust resistance in wheat (*Triticum aestivum*) typically signals the emergence of a new race (Peterson, 2001), 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 (Bonos et al., 2006), but this prediction has not yet been tested. Pathotype specificity has been demonstrated for different isolates of *P. graminis* in perennial ryegrass (*Lolium perenne*) turf (Pfender, 2009) and is suspected for isolates of *P. coronata* pathogenic to this species (Kimbeng, 1999, Potter et al., 1990, Studer et al., 2007). 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 (Cummins, 1971) 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 (Smiley et al., 2005). The number of accepted rust species varies between studies, since morphological characteristics and host plant association (Smiley et al., 2005) are the primary mode of identifying turfgrass rusts,
and interpretation of these characters can vary depending on the taxonomic treatment used. The use of morphology for rust identification is complicated, as subtle differences such as teliospore size, shape, and pedicel type (Maier et al., 2007) 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 species can also be problematic. Complete lifecycles are unknown for many graminicolous rusts, making it impossible to fully describe host ranges (Aime et al., 2006), and cross-infectivity between hosts has been reported in inoculation studies (Pfender, 2001). Despite the uncertainty associated with the use of host identity and spore phenotype for rust species identification, these techniques are routinely used in the field, since 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 (Anikster et al., 2004, Crouch and Szabo, 2011, Zambino and Szabo, 1993), 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 complete non-coding ITS 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 and/or race associations. We surveyed a range of diseased turfgrass hosts to assess molecular variability of the ITS region in several commercially important turfgrass species. As an alternative to using
morphological characteristics and host plant association for turfgrass rust identification, this sequence data was then used to generate a real-time 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 and/or teliospores were collected from thirteen states in the U.S. (Figure 1), 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 used for molecular analyses.

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 (Barletsville, OK) on the medium setting for six minutes. Extraction buffer (2X STE [1X=100 mM NaCl, 50 mM Tris pH 8.0, 1 mM EDTA]; 2% SDS; 2% β-mercaptoethanol) was added to create a slurry that was mixed with 1x vol. phenol. After ethanol precipitation of the supernatant, pelleted DNA was resuspended in TE buffer (1M Tris [pH 8.0]; 0.5M EDTA) and incubated at
37°C with RNaseA (1 μg/mL) for 30 minutes. 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).

ITS primers 4 and 5 (White et al., 1990) were used to PCR amplify the complete nuclear ribosomal internal transcribed spacer (ITS) 1, 5.8s, and ITS2, including partial flanking regions of the 18s and 28s ribosomal DNA. All samples were PCR amplified in a GeneAmp Thermocycler (Applied Biosystems, Foster City, CA) using cycling conditions previously described (Crouch et al., 2006). Amplicons were excised from 0.8% agarose gels, purified using the GeneClean III Kit (QBiogene, Irvine, CA), ligated into pGEM-T Easy vectors (Promega, Madison, WI), and transformed into α-Select Gold Efficiency competent cells (BioLine, Taunton, MA). 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 from 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).

*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. *Puccinia hordei* and *P. recondita* were used as outgroup taxa for an analysis inclusive of all sampled taxa (Abbasi et al., 2005, Zambino and Szabo, 1993). *P.
*graminis* was used as the outgroup for the analysis of a dataset comprised entirely of *P. coronata* sequences (Zambino and Szabo, 1993).

Sequences were aligned using Clustal X2 (Larkin et al., 2007), 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 (Swofford, 2002) to identify duplicate sequences, with one representative of each group retained for phylogenetic analysis. Phylogenetic analysis was performed using BEAST v1.4.8 (Drummond and Rambaut, 2007) with a GTR + I + γ model, empirical base frequencies, 4 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 rust 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 (Available from http://beast.bio.ed.ac.uk/Tracer.), with the first 10,000 trees discarded from the complete rust analysis and the first 1,000 trees from the *P. coronata* analysis. TreeAnnotator v1.4.8 (Available from 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 (Available from 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 turfgrass rust 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 Black Hole Quencher 2 (BHQ-2) (IDT, Coralville, IA), allowing for multiplexing with PuSTR-ITS1. PuCR-ITS2 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 Iowa Black Fluorescent Quencher (IBFQ) (IDT, Coralville, IA). An additional internal quencher, ZEN (IDT, Coralville, IA), 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 (Barnes and Szabo, 2007), respectively, were synthesized for comparison with the turfgrass rust primers/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 218/219bp or 198bp region amplified in the real-time assays for each rust species in the pUC57 plasmid vector (GeneWiz, Inc., South Plainfield, NJ). By synthesizing each turfgrass rust species’ ITS amplicon in their own pUC57 plasmid vector, we ensured that positive controls did not contain mixed DNA concentrations of the three turfgrass rust species, as may occur when using turfgrass rust samples collected in the field as positive controls. To avoid overloading the reaction with plasmid template DNA, 15 ng/µl positive control plasmid DNA was mixed with an equal part of sheared salmon sperm DNA (Invitrogen, Carlsbad, CA). A previously designed real-time PCR primer/probe set, StdLSU1, specific to the fungal large subunit region served as an internal standard control (Barnes and Szabo, 2007).

Real-time PCR reactions were performed using a Cepheid SmartCycler (Cepheid, Sunnyvale, CA) in 25 µl 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, Foster City, CA). Optimal cycling conditions were determined by using concentration gradients of 10 µM to 1 µM of primers and probes at different annealing temperatures (54°C to 60°C), and the cycle with the earliest detection was retained for all further analysis. The final reaction volumes contained 5 µl of DNA and 20 µl of Cepheid master mix, with 10 µM of both primers and 1 µM of the 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 rust samples against each probe in a 96-well format, with each run replicated three times (Table 3). Each rust sample was also screened using probes Pg FAM 1 and Ps FAM 2 (Barnes and Szabo, 2007), designed for detection of *P. graminis f. sp. tritici* and *P. striiformis* in wheat. Probes were considered specific if cycle threshold values were zero for non-target DNA and water controls.

Assay sensitivity was determined by generating 10-fold dilutions of genomic rust DNA until target DNA could not be detected, with concentrations between 3 pg/µl and 40 ng/µl. Assays were further examined by preparing reactions containing DNA extracted from 50, 100, 150, 200, and 300 individual rust spores for each of the rust 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 the fall of 2008, 66 turfgrass samples exhibiting symptoms of rust disease were collected from thirteen U. S. states (Figure 1) and four countries (Table 1). Phylogenetic analysis of the ITS region identified *P. coronata*, *P. graminis*, and *P. striiformis* (Figure 2) 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; Figures 2 and 3). Both *P. coronata* and *P. graminis* were identified from a wide range of turfgrass hosts. *P. coronata* was found on seven different grass hosts: brome grass (*Bromus* sp.), tufted hair grass (*Deschampsia sp.*), Kentucky bluegrass, perennial ryegrass, reedgrass (*Calamagrostis sp.*), tall fescue (*Festuca arundinacea*), and zoysia grass (*Zoysia sp.*) (Table 1). In contrast, *P. graminis* was identified on a much smaller host range, consisting of tall fescue and Kentucky bluegrass. *P. striiformis* was identified on only two grass hosts, perennial ryegrass and annual bluegrass (*Poa annua*).

All three rust fungal species formed well supported monophyletic groups, with posterior probabilities ≥ 0.99 (Figure 2). Within the three rust species clades, turfgrass isolates clustered together and were separate from non-turfgrass rust fungi (posterior probabilities ≥ 0.91). Within these divisions, there was no segregation based on geography, although sampling was not designed to test geographic distribution. *P. 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 isolates within *P. coronata* and *P. graminis* formed well-supported sub-groupings within their respective clades (Figures 2 and 3); however, there were only four of these sub-groups. Overall, outside of the division between cereal and turf isolates in each of the three species, little intraspecific variation was observed.

*Real-time PCR 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, as the pustules exhibit an easily distinguishable color that has been thought to separate the fungus from *P. coronata* and *P. striiformis* (Smiley et al., 2005). Phenotypic identification of *P. graminis* from turf has also been used in conjunction with host association criteria, since the fungus was expected to be found in association with bluegrass, but not ryegrass hosts (Smiley et al., 2005, Vargas, 2005). Inaccurate identification of turfgrass rust fungi has been most commonly associated with the differentiation between *P. coronata* and *P. striiformis*, as 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 (Liu and Hambleton, 2010). In the present study, the application of host plant association and spore phenotype characters incorrectly identified 42% of the turfgrass rust samples (Table 4), with over half of these misidentifications coming from samples of rust fungi on Kentucky bluegrass. Using classical field identification techniques, 58% of the rust 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.

Real-time PCR has been previously shown to be effective for discrimination and detection of several rust fungi, including *Phakopsora pachyrhizi*, *P. graminis* f.sp. *tritici*, *P. kuehnii*, *P. melanocephala*, *P. polysora*, *P. recondita*, *P. sorghi*, *P. striiformis* (from cereals), and *P. triticina* (Barnes and Szabo, 2007, Barnes et al., 2009, Crouch and Szabo, 2011, Glynn et al., 2010, Jin et al., 2010). We attempted to use 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 (Barnes and Szabo, 2007); however, the wheat rust fungal assays produced variable results when used to evaluate this group (Table 3). 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 (Table 3). However, neither probe consistently identified the different turf rust species correctly. The existing Pg-FAM-1 probe sequence contained one unique SNP (single nucleotide polymorphism) 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* turfgrass fungi. 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 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 turf rust species. Two probes, PuSTR-ITS1 and PuSTM-ITS1, were designed within the ITS1 region to detect *P. striiformis* and *P. graminis*. The probe region differed between the three turfgrass rust fungi by at least 2 SNPs. Figure 4 illustrates the primer and probe binding sites for PuSTR-ITS1 and PuSTM-ITS1 in both turfgrass isolates and representative cereal isolates. 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* (Figure 5). When used to screen rust 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 Table 3 and Figure 8 A-C. Non-target DNA and negative controls produced C_T values equal to zero. Detection using PuSTR-ITS1 began at 50 spores (C_T = 32.74), PuSTM-ITS1 at 150 spores (C_T = 36.62) and PuCR-ITS2 at 150 spores (C_T = 34.42). Assay sensitivity determined by serial dilutions of genomic rust DNA showed the lowest levels of
detection to be between 1 to 9 pg (Figure 6). Positive controls that were synthesized in the pUC57 plasmid vector repeatedly produced fluorescence early in the PCR cycle (Figure 8), with C_T values ranging from 20-25. Biological samples generated fluorescence curves later in the cycle, with C_T values ranging between 27 and 38 (Figure 8) and increased with decreasing DNA concentrations. Non-specific positive reactions were observed from four targets – OH-1A/B and OH-8A/B (Table 3), however, additional sequencing of clones with ITS inserts 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 cycle threshold value (C_T = 19.73 and 18.11, respectively) comparable to non-multiplexed reactions (Figure 7). 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 cycle threshold values of 21.60 and 23.97 (Figure 7).
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 (Cummins, 1971); 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.

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 (1993). Our data showed no pattern of segregation based on locale in any of the clades, suggesting that turfgrass populations of rust fungi are widespread and highly mobile, as is known to occur in the wheat stem rust pathosystem (Kolmer, 2005). Population-scale investigations are needed to test this idea further, as the sampling and molecular markers used in the present research were not designed to address distribution patterns.

In our analyses, subspecific groups corresponding with 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 (Abbasi, 2005). 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 (2005) put forth the idea that these sub-specific groups might be re-categorized as two distinct species, but, to date, this possibility has not been robustly tested through comprehensive sampling of the fungus and multi-locus phylogenetic study 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 (Bahri et al., 2009, Chen et al., 2009, Duan et al., 2010, Hovmoller et al., 2008, Jafary et al., 2006). The recent description of four strongly supported monophyletic lineages within *P. striiformis* supports division of the species to *P. striiformis* sensu stricto on Triticeae, *P. pseudostriiformis* on bluegrass, *P. striiformoides* on orchard grasses (*Dactylis* sp.), and *P. gansensis* on needlegrass (*Achnatherum* sp.) (Abbasi et al., 2004, Liu and Hambleton, 2010). Though sampling within the *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 Triticeae, orchard grass, and cultivated turfgrass hosts, consistent with the groupings by Liu and Hambleton (2010). While 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*) (Jin et al., 2010), 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 comprised of samples from non-turfgrass hosts, and a second group largely composed of rust fungal samples from turfgrass hosts (Figure 3). In the turfgrass clade, additional subspecific clustering was also observed, but these groups were not supported 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 = 0.00000) to at least one isolate from the sample (data not shown).

**Real-time PCR for the accurate and quick identification of turfgrass rust species**

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 rust DNA can be detected through this protocol – as little as ~1-9 pg, or 50-150 spores...
with an accurate identification provided from DNA samples in only 30 minutes. 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 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 rusts. 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.

*Puccinia 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 (Smiley et al., 2005); although recent research has shown that orientation of the sorus is an unreliable feature (Liu and Hambleton, 2010). 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 (Smiley et al., 2005, Vargas, 2005). 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, since 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 (Bonos et al., 2006); a finding that is consistent with the long-term, stable race structure of *P. graminis* in the U.S. since the eradication of barberry across the Great Plains. Instead, our data supports 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 U.K., Poland, China and the Czech Republic; however, this is the first published report of this host/fungus association from the western hemisphere (Farr and Rossman, 2010). Nevertheless, *P. coronata* has occurred on Kentucky bluegrass in North America in the past, as our observations of herbarium specimens revealed the presence of the fungus at least as far back as the 19th century (e.g., BPI060420 in the U.S. National Fungal Collections from Missouri, collected 30-Oct-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*) (Figure 3). Because *P. coronata* is highly variable (Cummins, 1971), 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*) (Jin and Steffesnson, 1992) and smooth brome (*Bromus inermis*) (Delgado et al., 2001).

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*) 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 U.S., with mean virulence significantly increasing during this period (Carson, 2008). Such a scenario in Kentucky bluegrass could have far reaching implications for the turfgrass industry, but remains to be validated.
REFERENCES


Table 1. Sources of rust fungal samples used in this study

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Host Species</th>
<th>Origin</th>
<th>Accession Number</th>
<th>Original Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR-1</td>
<td><em>Arrhenatherum elatius</em></td>
<td>Czech Republic</td>
<td>DQ355443&lt;sup&gt;b&lt;/sup&gt;</td>
<td>43</td>
</tr>
<tr>
<td>MNCL-1</td>
<td><em>Avena sativa</em></td>
<td>Minnesota- USA</td>
<td>AY114290&lt;sup&gt;b&lt;/sup&gt;</td>
<td>43</td>
</tr>
<tr>
<td>UK-2&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>Avena sp.</em></td>
<td>United Kingdom</td>
<td>GU598105</td>
<td>This study</td>
</tr>
<tr>
<td>UK-5&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>Avena sp.</em></td>
<td>United Kingdom</td>
<td>GU598106</td>
<td>This study</td>
</tr>
<tr>
<td>UKF-12&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>Avena sp.</em></td>
<td>United Kingdom</td>
<td>GU598109</td>
<td>This study</td>
</tr>
<tr>
<td>SLV-1</td>
<td><em>Bromus erectus</em></td>
<td>Slovakia</td>
<td>DQ355449&lt;sup&gt;b&lt;/sup&gt;</td>
<td>43</td>
</tr>
<tr>
<td>CR-2</td>
<td><em>Bromus erectus</em></td>
<td>Czech Republic</td>
<td>DQ355450&lt;sup&gt;b&lt;/sup&gt;</td>
<td>43</td>
</tr>
<tr>
<td>MNCL-9</td>
<td><em>Bromus inermis</em></td>
<td>USA</td>
<td>DQ355448&lt;sup&gt;b&lt;/sup&gt;</td>
<td>43</td>
</tr>
<tr>
<td>MNCL-10</td>
<td><em>Bromus inermis</em></td>
<td>USA</td>
<td>DQ355446&lt;sup&gt;b&lt;/sup&gt;</td>
<td>43</td>
</tr>
<tr>
<td>MI-1&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>Bromus sp.</em></td>
<td>Michigan-USA</td>
<td>GU598069</td>
<td>This study</td>
</tr>
<tr>
<td>UKF-8&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>Calamagrostis sp.</em></td>
<td>United Kingdom</td>
<td>GU598107</td>
<td>This study</td>
</tr>
<tr>
<td>UKF-10&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>Calamagrostis sp.</em></td>
<td>United Kingdom</td>
<td>GU598108</td>
<td>This study</td>
</tr>
<tr>
<td>NJA-24&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>Deschampsia sp.</em></td>
<td>New Jersey- USA</td>
<td>GU598078</td>
<td>This study</td>
</tr>
<tr>
<td>MNCL-2</td>
<td><em>Elytrigia repens</em></td>
<td>Minnesota- USA</td>
<td>DQ414723&lt;sup&gt;b&lt;/sup&gt;</td>
<td>43</td>
</tr>
<tr>
<td>MNCL-3</td>
<td><em>Elytrigia repens</em></td>
<td>Minnesota- USA</td>
<td>DQ414724&lt;sup&gt;b&lt;/sup&gt;</td>
<td>43</td>
</tr>
<tr>
<td>MS-1&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>Festuca arundinacea</em></td>
<td>Mississippi- USA</td>
<td>GU598056</td>
<td>This study</td>
</tr>
<tr>
<td>NJA-66&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>Festuca arundinacea</em></td>
<td>New Jersey- USA</td>
<td>GU598047</td>
<td>This study</td>
</tr>
<tr>
<td>NJA-67&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>Festuca arundinacea</em></td>
<td>New Jersey- USA</td>
<td>GU598048</td>
<td>This study</td>
</tr>
<tr>
<td>NJA-9&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>Festuca sp.</em></td>
<td>New Jersey- USA</td>
<td>GU598072</td>
<td>This study</td>
</tr>
<tr>
<td>IR-1</td>
<td><em>Holcus lanatus</em></td>
<td>Ireland</td>
<td>DQ355444&lt;sup&gt;b&lt;/sup&gt;</td>
<td>43</td>
</tr>
<tr>
<td>UO-1</td>
<td><em>Hordeum vulgare</em></td>
<td>unknown</td>
<td>DQ355454&lt;sup&gt;b&lt;/sup&gt;</td>
<td>43</td>
</tr>
<tr>
<td>CH-1&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>Lolium perenne</em></td>
<td>Chile</td>
<td>GU598061</td>
<td>This study</td>
</tr>
<tr>
<td>CH-3&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>Lolium perenne</em></td>
<td>Chile</td>
<td>GU598062</td>
<td>This study</td>
</tr>
<tr>
<td>CT-3&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>Lolium perenne</em></td>
<td>Connecticut- USA</td>
<td>GU598059</td>
<td>This study</td>
</tr>
<tr>
<td>MA-1&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>Lolium perenne</em></td>
<td>Massachusetts- USA</td>
<td>GU598063</td>
<td>This study</td>
</tr>
<tr>
<td>MA-2&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>Lolium perenne</em></td>
<td>Massachusetts- USA</td>
<td>GU598064</td>
<td>This study</td>
</tr>
<tr>
<td>MA-3&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>Lolium perenne</em></td>
<td>Massachusetts- USA</td>
<td>GU598065</td>
<td>This study</td>
</tr>
<tr>
<td>NJC-7&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>Lolium perenne</em></td>
<td>New Jersey- USA</td>
<td>GU598079</td>
<td>This study</td>
</tr>
<tr>
<td>NJCC-31&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>Lolium perenne</em></td>
<td>New Jersey- USA</td>
<td>GU598083</td>
<td>This study</td>
</tr>
<tr>
<td>NJCC-33&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>Lolium perenne</em></td>
<td>New Jersey- USA</td>
<td>GU598084</td>
<td>This study</td>
</tr>
<tr>
<td>CR-3</td>
<td><em>Lolium perenne</em></td>
<td>Czech Republic</td>
<td>DQ355441&lt;sup&gt;b&lt;/sup&gt;</td>
<td>43</td>
</tr>
<tr>
<td>UK-3&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>Lolium perenne</em></td>
<td>United Kingdom</td>
<td>GU598049</td>
<td>This study</td>
</tr>
<tr>
<td>UKF-13&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>Lolium perenne</em></td>
<td>United Kingdom</td>
<td>GU598110</td>
<td>This study</td>
</tr>
<tr>
<td>UKF-14&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>Lolium perenne</em></td>
<td>United Kingdom</td>
<td>GU598111</td>
<td>This study</td>
</tr>
<tr>
<td>NJNB-37&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>Poa pratensis</em></td>
<td>New Jersey- USA</td>
<td>GU598091</td>
<td>This study</td>
</tr>
<tr>
<td>NJCC-42&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>Poa pratensis</em></td>
<td>New Jersey- USA</td>
<td>GU598085</td>
<td>This study</td>
</tr>
<tr>
<td>NJE-50&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>Poa pratensis</em></td>
<td>New Jersey- USA</td>
<td>GU598086</td>
<td>This study</td>
</tr>
<tr>
<td>MEG-1&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>Poa pratensis</em></td>
<td>Maine- USA</td>
<td>GU598068</td>
<td>This study</td>
</tr>
<tr>
<td>WY-1&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>Poa pratensis</em></td>
<td>Wyoming- USA</td>
<td>GU598112</td>
<td>This study</td>
</tr>
<tr>
<td>NC-2&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>Poa pratensis</em></td>
<td>North Carolina- USA</td>
<td>GU598070</td>
<td>This study</td>
</tr>
<tr>
<td>OH-3&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>Poa pratensis</em></td>
<td>Ohio- USA</td>
<td>GU598098</td>
<td>This study</td>
</tr>
<tr>
<td>OH-4&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>Poa pratensis</em></td>
<td>Ohio- USA</td>
<td>GU598099</td>
<td>This study</td>
</tr>
<tr>
<td>OH-5&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>Poa pratensis</em></td>
<td>Ohio- USA</td>
<td>GU598100</td>
<td>This study</td>
</tr>
<tr>
<td>MA-4&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>Poa pratensis</em></td>
<td>Massachusetts- USA</td>
<td>GU598066</td>
<td>This study</td>
</tr>
<tr>
<td>Isolate</td>
<td>Host Species</td>
<td>Origin</td>
<td>Accession Number</td>
<td>Original Reference</td>
</tr>
<tr>
<td>----------</td>
<td>-------------------</td>
<td>-------------------</td>
<td>------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>MA-5</td>
<td>Poa pratensis</td>
<td>Massachusetts- USA</td>
<td>GU598067</td>
<td>This study</td>
</tr>
<tr>
<td>NJA-19</td>
<td>Poa pratensis</td>
<td>New Jersey- USA</td>
<td>GU598075</td>
<td>This study</td>
</tr>
<tr>
<td>NJA-20</td>
<td>Poa pratensis</td>
<td>New Jersey- USA</td>
<td>GU598076</td>
<td>This study</td>
</tr>
<tr>
<td>NJA-21</td>
<td>Poa pratensis</td>
<td>New Jersey- USA</td>
<td>GU598077</td>
<td>This study</td>
</tr>
<tr>
<td>NJA-23</td>
<td>Poa pratensis</td>
<td>New Jersey- USA</td>
<td>GU598054</td>
<td>This study</td>
</tr>
<tr>
<td>NJC-28</td>
<td>Poa pratensis</td>
<td>New Jersey- USA</td>
<td>GU598081</td>
<td>This study</td>
</tr>
<tr>
<td>NJC-29</td>
<td>Poa pratensis</td>
<td>New Jersey- USA</td>
<td>GU598082</td>
<td>This study</td>
</tr>
<tr>
<td>NJHP-32</td>
<td>Poa pratensis</td>
<td>New Jersey- USA</td>
<td>GU598090</td>
<td>This study</td>
</tr>
<tr>
<td>NJT-4</td>
<td>Poa pratensis</td>
<td>New Jersey- USA</td>
<td>GU598092</td>
<td>This study</td>
</tr>
<tr>
<td>OH-1B</td>
<td>Poa pratensis</td>
<td>Ohio- USA</td>
<td>GU598097</td>
<td>This study</td>
</tr>
<tr>
<td>OH-8B</td>
<td>Poa pratensis</td>
<td>Ohio- USA</td>
<td>GU598052</td>
<td>This study</td>
</tr>
<tr>
<td>MNCL-4</td>
<td>Rhamnus catharticus</td>
<td>Minnesota- USA</td>
<td>DQ355445</td>
<td>43</td>
</tr>
<tr>
<td>MNCL-5</td>
<td>Rhamnus catharticus</td>
<td>Minnesota- USA</td>
<td>DQ355447</td>
<td>43</td>
</tr>
<tr>
<td>MNCL-6</td>
<td>Rhamnus catharticus</td>
<td>Minnesota- USA</td>
<td>DQ355442</td>
<td>43</td>
</tr>
<tr>
<td>MNCL-7</td>
<td>Rhamnus catharticus</td>
<td>Minnesota- USA</td>
<td>DQ355452</td>
<td>43</td>
</tr>
<tr>
<td>SLV-2</td>
<td>Rhamnus saxatilis</td>
<td>Slovakia</td>
<td>DQ355451</td>
<td>43</td>
</tr>
<tr>
<td>NJC-8</td>
<td>Zoysia sp.</td>
<td>New Jersey- USA</td>
<td>GU598080</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Puccinia graminis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CR-4</td>
<td>Anthroxanthum sp.</td>
<td>Czech Republic</td>
<td>DQ417386</td>
<td>7</td>
</tr>
<tr>
<td>SW-1</td>
<td>Avena sativa</td>
<td>Switzerland</td>
<td>DQ417391</td>
<td>7</td>
</tr>
<tr>
<td>SW-2</td>
<td>Dactylis glomerata</td>
<td>Switzerland</td>
<td>DQ417390</td>
<td>7</td>
</tr>
<tr>
<td>WI-1</td>
<td>Elymus trachycaulus</td>
<td>Wisconsin- USA</td>
<td>DQ417381</td>
<td>7</td>
</tr>
<tr>
<td>NJF-45</td>
<td>Festuca arundinacea</td>
<td>New Jersey- USA</td>
<td>GU598087</td>
<td>This study</td>
</tr>
<tr>
<td>NJH-48</td>
<td>Festuca arundinacea</td>
<td>New Jersey- USA</td>
<td>GU598088</td>
<td>This study</td>
</tr>
<tr>
<td>NJH-49</td>
<td>Festuca arundinacea</td>
<td>New Jersey- USA</td>
<td>GU598089</td>
<td>This study</td>
</tr>
<tr>
<td>OR-1</td>
<td>Festuca arundinacea</td>
<td>Oregon- USA</td>
<td>DQ417385</td>
<td>7</td>
</tr>
<tr>
<td>OR-2</td>
<td>Lolium perenne</td>
<td>Oregon- USA</td>
<td>DQ417384</td>
<td>7</td>
</tr>
<tr>
<td>UO-2</td>
<td>Lolium perenne</td>
<td>unknown</td>
<td>DQ355455</td>
<td>41</td>
</tr>
<tr>
<td>UO-3</td>
<td>Lolium sp.</td>
<td>unknown</td>
<td>DQ460726</td>
<td>23</td>
</tr>
<tr>
<td>MNCL-8</td>
<td>Phileum pretense</td>
<td>Minnesota- USA</td>
<td>DQ417392</td>
<td>7</td>
</tr>
<tr>
<td>NJT-5</td>
<td>Poa pratensis</td>
<td>New Jersey- USA</td>
<td>GU598093</td>
<td>This study</td>
</tr>
<tr>
<td>NYP-1</td>
<td>Poa pratensis</td>
<td>New York- USA</td>
<td>GU598095</td>
<td>This study</td>
</tr>
<tr>
<td>NJGM-39</td>
<td>Poa pratensis</td>
<td>New Jersey- USA</td>
<td>GU598053</td>
<td>This study</td>
</tr>
<tr>
<td>ONCA-1</td>
<td>Poa pratensis</td>
<td>Canada</td>
<td>GU598103</td>
<td>This study</td>
</tr>
<tr>
<td>NE-1</td>
<td>Poa pratensis</td>
<td>Nebraska- USA</td>
<td>GU598071</td>
<td>This study</td>
</tr>
<tr>
<td>NC-1</td>
<td>Poa pratensis</td>
<td>North Carolina- USA</td>
<td>GU598070</td>
<td>This study</td>
</tr>
<tr>
<td>OH-6</td>
<td>Poa pratensis</td>
<td>Ohio- USA</td>
<td>GU598101</td>
<td>This study</td>
</tr>
<tr>
<td>OH-7</td>
<td>Poa pratensis</td>
<td>Ohio- USA</td>
<td>GU598102</td>
<td>This study</td>
</tr>
<tr>
<td>MNM-1</td>
<td>Poa pratensis</td>
<td>Minnesota- USA</td>
<td>GU598058</td>
<td>This study</td>
</tr>
<tr>
<td>NJA-53</td>
<td>Poa pratensis</td>
<td>New Jersey- USA</td>
<td>GU598057</td>
<td>This study</td>
</tr>
<tr>
<td>NJA-54</td>
<td>Poa pratensis</td>
<td>New Jersey- USA</td>
<td>GU598060</td>
<td>This study</td>
</tr>
<tr>
<td>NJA-17</td>
<td>Poa pratensis</td>
<td>New Jersey- USA</td>
<td>GU598073</td>
<td>This study</td>
</tr>
<tr>
<td>NJA-18</td>
<td>Poa pratensis</td>
<td>New Jersey- USA</td>
<td>GU598074</td>
<td>This study</td>
</tr>
<tr>
<td>OH-1A</td>
<td>Poa pratensis</td>
<td>Ohio- USA</td>
<td>GU598096</td>
<td>This study</td>
</tr>
<tr>
<td>OH-8A</td>
<td>Poa pratensis</td>
<td>Ohio- USA</td>
<td>GU598051</td>
<td>This study</td>
</tr>
<tr>
<td>SW-3</td>
<td>Poa pratensis</td>
<td>Switzerland</td>
<td>DQ417389</td>
<td>7</td>
</tr>
<tr>
<td>KS-1</td>
<td>Triticum aestivum</td>
<td>Kansas- USA</td>
<td>DQ417374</td>
<td>7</td>
</tr>
<tr>
<td>OHS-1</td>
<td>Triticum aestivum</td>
<td>Ohio- USA</td>
<td>DQ417375</td>
<td>7</td>
</tr>
<tr>
<td>MX-1</td>
<td>Triticum aestivum</td>
<td>Mexico</td>
<td>DQ417377</td>
<td>7</td>
</tr>
<tr>
<td>OR-3</td>
<td>Triticum aestivum</td>
<td>Oregon- USA</td>
<td>DQ417380</td>
<td>7</td>
</tr>
<tr>
<td>Isolate</td>
<td>Host Species</td>
<td>Origin</td>
<td>Accession Number</td>
<td>Original Reference</td>
</tr>
<tr>
<td>---------</td>
<td>------------------</td>
<td>----------------</td>
<td>------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>SW-4</td>
<td>Triticum aestivum</td>
<td>Switzerland</td>
<td>DQ417387</td>
<td>7</td>
</tr>
<tr>
<td>SD-1</td>
<td>Triticum aestivum</td>
<td>South Dakota- USA</td>
<td>DQ417383</td>
<td>7</td>
</tr>
<tr>
<td>UO-4</td>
<td>Triticum aestivum</td>
<td>unknown</td>
<td>AY114289</td>
<td>43</td>
</tr>
<tr>
<td><strong>Puccinia striiformis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MNC-1</td>
<td>Berberis chinensis</td>
<td>Minnesota-USA</td>
<td>GU382672</td>
<td>21</td>
</tr>
<tr>
<td>MNC-5</td>
<td>Berberis chinensis</td>
<td>Minnesota-USA</td>
<td>GQ457305</td>
<td>21</td>
</tr>
<tr>
<td>CR-5</td>
<td>Dactylis glomerata</td>
<td>Czech Republic</td>
<td>DQ417403</td>
<td>7</td>
</tr>
<tr>
<td>WA-1</td>
<td>Hordeum vulgare</td>
<td>Washington- USA</td>
<td>DQ417408</td>
<td>7</td>
</tr>
<tr>
<td>CA-1</td>
<td>Hordeum vulgare</td>
<td>California- USA</td>
<td>DQ417402</td>
<td>7</td>
</tr>
<tr>
<td>PA-2</td>
<td>Lolium perenne</td>
<td>Pennsylvania- USA</td>
<td>GU598104</td>
<td>This study</td>
</tr>
<tr>
<td>NYH-4</td>
<td>Lolium perenne</td>
<td>New York- USA</td>
<td>GU598094</td>
<td>This study</td>
</tr>
<tr>
<td>AU-1</td>
<td>Poa annua</td>
<td>Australia</td>
<td>GU598050</td>
<td>This study</td>
</tr>
<tr>
<td>GA-1</td>
<td>Poa pratensis</td>
<td>Georgia-USA</td>
<td>DQ417407</td>
<td>7</td>
</tr>
<tr>
<td>MNC-2</td>
<td>Triticum aestivum</td>
<td>Minnesota-USA</td>
<td>GU382671</td>
<td>21</td>
</tr>
<tr>
<td>MNC-3</td>
<td>Triticum aestivum</td>
<td>Minnesota-USA</td>
<td>GU382673</td>
<td>21</td>
</tr>
<tr>
<td>MNC-4</td>
<td>Triticum aestivum</td>
<td>Minnesota-USA</td>
<td>DQ417394</td>
<td>7</td>
</tr>
<tr>
<td>WA-2</td>
<td>Triticum aestivum</td>
<td>Washington- USA</td>
<td>DQ417395</td>
<td>7</td>
</tr>
<tr>
<td>DNM-1</td>
<td>Triticum aestivum</td>
<td>Denmark</td>
<td>DQ417397</td>
<td>7</td>
</tr>
<tr>
<td>DNM-2</td>
<td>Triticum aestivum</td>
<td>Denmark</td>
<td>DQ417404</td>
<td>7</td>
</tr>
<tr>
<td>TX-1</td>
<td>Triticum aestivum</td>
<td>Texas- USA</td>
<td>DQ417405</td>
<td>7</td>
</tr>
<tr>
<td>TX-2</td>
<td>Triticum aestivum</td>
<td>Texas- USA</td>
<td>DQ417406</td>
<td>7</td>
</tr>
<tr>
<td><strong>Puccinia hordei</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UO-5</td>
<td>Hordeum vulgare</td>
<td>unknown</td>
<td>AY187089</td>
<td>4</td>
</tr>
<tr>
<td><strong>Puccinia recondita</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UO-6</td>
<td>Hordeum vulgare</td>
<td>unknown</td>
<td>EU014045</td>
<td>3</td>
</tr>
</tbody>
</table>

*a* Rust fungal samples sequenced in this study and used for verification of real-time PCR assays

*b* Previous GenBank submissions
Table 2. PCR primers and hydrolysis probes developed in this study.

<table>
<thead>
<tr>
<th>Primer / Probe</th>
<th>Description</th>
<th>Sequence (5'-3')</th>
<th>Location (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FrITS2Cr</td>
<td><em>P. coronata</em> forward ITS2</td>
<td>TTTGTGGATGTTGAGTGTTC</td>
<td>465-485f</td>
</tr>
<tr>
<td>RrITS2Cr</td>
<td><em>P. coronata</em> reverse ITS2</td>
<td>TCCCACCTGATTGAGTCTT</td>
<td>644-663f</td>
</tr>
<tr>
<td>FrITS1Pu</td>
<td><em>P. graminis</em> &amp; <em>P. striiformis</em> forward ITS1</td>
<td>CCTCGGAAGGATCATTTT</td>
<td>37-56h</td>
</tr>
<tr>
<td>RrITS1Pu</td>
<td><em>P. graminis</em> &amp; <em>P. striiformis</em> reverse ITS1</td>
<td>TTTGGTTAACATTGTTAAC</td>
<td>230-255h</td>
</tr>
<tr>
<td>PuCR-ITS2</td>
<td><em>Puccinia coronata</em> Probe</td>
<td>FAM&lt;sup&gt;a&lt;/sup&gt;-TACTTGCCA-ZEN&lt;sup&gt;b&lt;/sup&gt;-CTTTTGAAAGGAGGGA-IaBk&lt;sup&gt;c&lt;/sup&gt;</td>
<td>587-612h</td>
</tr>
<tr>
<td>PuSTM-ITS1</td>
<td><em>Puccinia graminis</em> Probe</td>
<td>Cy3&lt;sup&gt;d&lt;/sup&gt;-TTAGAGTCACCTTTATTTGTCGCTCACTCTCT-BHQ2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>64-95h</td>
</tr>
<tr>
<td>PuSTR-ITS1</td>
<td><em>Puccinia striiformis</em> Probe</td>
<td>FAM&lt;sup&gt;a&lt;/sup&gt;-CGTAACCTCTTTATTTGATGCGATTACCCTCCC-BHQ1&lt;sup&gt;f&lt;/sup&gt;</td>
<td>160-194h</td>
</tr>
</tbody>
</table>

<sup>a</sup> FAM = 6-carboxy-fluorescein fluorescent reporter dye (IDT, Coralville, IA)
<sup>b</sup> ZEN = internal quencher to enhance specificity (IDT, Coralville, IA)
<sup>c</sup> IBFQ = Iowa Black Fluorescent Quencher (IDT, Coralville, IA)
<sup>d</sup> Cy3 = Cyanine 3 fluorescent reporter dye (IDT, Coralville, IA)
<sup>e</sup> BHQ-2 = Black Hole Quencher 2 (IDT, Coralville, IA)
<sup>f</sup> BHQ-1 = Black Hole Quencher 1 (IDT, Coralville, IA)
<sup>g</sup> Primer/probe set base pair position in ITS2
<sup>h</sup> Primer/probe set base pair position in ITS1
Table 3. Results of real-time PCR experiments using different probes to screen fungal samples. All fungal samples were screened against each probe three times, where + = positive and - = negative results. Probes designed in this study were specific to their respective turfgrass rust species.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>PuCR-ITS²</th>
<th>PuSTM-ITS¹</th>
<th>PuSTR ITS-1³</th>
<th>StdLSU¹⁴</th>
<th>Pg FAM-1⁵</th>
<th>Ps FAM-2⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Puccinia coronata</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UK-2</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>UK-5</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>UKF-12</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>MI-1</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>UKF-8</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>UKF-10</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>NJA-24</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>MS-1</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>NJA-66</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>NJA-67</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>NJA-9</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>CH-1</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>CH-3</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>CT-3</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>MA-1</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>MA-2</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>MA-3</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>NJC-7</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>NJCC-31</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>NJCC-33</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>UK-3</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>UKF-13</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>UKF-14</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>NINB-37</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>NJCC-42</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>NJE-50</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>MEG-1</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>WY-1</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>NC-2</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>OH-3</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>OH-4</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>OH-5</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>MA-4</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>MA-5</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>NJA-19</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>NJA-20</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>NJA-21</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>NJA-23</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>NIC-28</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>NIC-29</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>NHP-32</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>NIT-4</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>OH-1B</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>OH-8B</td>
<td>+ + +</td>
<td>- - +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>NJC-8</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>- - -</td>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>Isolates</td>
<td>PuCR-ITS2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>PuSTM-ITS1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>PuSTR ITS-1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>StdLSU1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Pg FAM-1&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Ps FAM-2&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>-------------------</td>
<td>------------------------</td>
<td>------------------------</td>
<td>-------------------------</td>
<td>---------------------</td>
<td>----------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td><em>Puccinia graminis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIH-48</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NIH-49</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NJI-5</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NYPI-1</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NJGM-39</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NE-1</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NC-1</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>OH-6</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>OH-7</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MNM-1</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NJA-53</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NJA-54</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NJA-17</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NJA-18</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>OH-1A</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>OH-8A</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NJF-45</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Puccinia striiformis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA-2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NYH-4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AU-1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Positive controls&lt;sup&gt;g&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUC57 <em>P. graminis</em></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pUC57 <em>P. coronata</em></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pUC57 <em>P. striiformis</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cereal rust isolates&lt;sup&gt;g&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. graminis</em></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>P. striiformis</em></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>P. coronata</em></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Negative controls&lt;sup&gt;h&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water blank 1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Water blank 2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Water blank 3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Colletotrichum cereale</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cryptonectria</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>P. graminis</em></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>P. striiformis</em></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>P. coronata</em></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Magnaporthe poae</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phytophthora infestans</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sclerotinia</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>homoeocarpa</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> PuCR-ITS2 = *Puccinia coronata* probe

<sup>b</sup> PuSTM-ITS1 = *P. graminis* probe

<sup>c</sup> PuSTR ITS-1 = *P. striiformis* probe

<sup>d</sup> StdLSU1 = standard internal control

<sup>e</sup> Pg FAM-1 = previously designed probe for the detection of *P. graminis* f.sp. *tritici* (8)

<sup>f</sup> Ps FAM-2 = previously designed probe for the detection of *P. striiformis* from cereal crops (8)
Positive controls were generated by synthesizing long oligonucleotides corresponding with the 218/219bp or 198bp region amplified in the real-time assays for each rust species in the pUC57 plasmid vector (GeneWiz, Inc., South Plainfield, NJ).

Cereal rust isolates obtained from United States Department of Agriculture-Agricultural Research Service, Cereal Disease Laboratory, St. Paul, MN

Internal negative water and fungal controls
Table 4. Comparison of turfgrass rust species identification using morphology, phenotype, host plant association, and sequence data techniques.

<table>
<thead>
<tr>
<th>Host Species</th>
<th>Morphological Characteristics</th>
<th>Probable Field I.D.</th>
<th>ITS Sequence Data I.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>UK-2</td>
<td>Avena sp.</td>
<td>P. coronata</td>
<td>P. coronata</td>
</tr>
<tr>
<td>UK-5</td>
<td>Avena sp.</td>
<td>P. coronata</td>
<td>P. coronata</td>
</tr>
<tr>
<td>UKF-12</td>
<td>Avena sp.</td>
<td>P. coronata</td>
<td>P. coronata</td>
</tr>
<tr>
<td>MI-1</td>
<td>Bromus sp.</td>
<td>P. coronata</td>
<td>P. coronata</td>
</tr>
<tr>
<td>UKF-8</td>
<td>Calamagrostis sp.</td>
<td>P. coronata</td>
<td>P. coronata</td>
</tr>
<tr>
<td>UKF-10</td>
<td>Calamagrostis sp.</td>
<td>P. coronata</td>
<td>P. coronata</td>
</tr>
<tr>
<td>NJA-24</td>
<td>Deschampsia sp.</td>
<td>P. coronata</td>
<td>P. coronata</td>
</tr>
<tr>
<td>MS-1</td>
<td>Festuca arundinacea</td>
<td>P. coronata / P. striiformis</td>
<td>P. coronata / P. striiformis</td>
</tr>
<tr>
<td>NJA-66</td>
<td>Festuca arundinacea</td>
<td>P. coronata</td>
<td>P. coronata</td>
</tr>
<tr>
<td>NJA-67</td>
<td>Festuca arundinacea</td>
<td>P. coronata</td>
<td>P. coronata</td>
</tr>
<tr>
<td>NJA-9</td>
<td>Festuca sp.</td>
<td>P. coronata</td>
<td>P. coronata</td>
</tr>
<tr>
<td>CH-1</td>
<td>Lolium perenne</td>
<td>P. coronata</td>
<td>P. coronata</td>
</tr>
<tr>
<td>CH-3</td>
<td>Lolium perenne</td>
<td>P. coronata</td>
<td>P. coronata</td>
</tr>
<tr>
<td>CT-3</td>
<td>Lolium perenne</td>
<td>P. coronata</td>
<td>P. coronata</td>
</tr>
<tr>
<td>MA-1</td>
<td>Lolium perenne</td>
<td>P. coronata / P. striiformis</td>
<td>P. coronata / P. striiformis</td>
</tr>
<tr>
<td>MA-2</td>
<td>Lolium perenne</td>
<td>P. coronata</td>
<td>P. coronata</td>
</tr>
<tr>
<td>MA-3</td>
<td>Lolium perenne</td>
<td>P. coronata / P. striiformis</td>
<td>P. coronata / P. striiformis</td>
</tr>
<tr>
<td>NJC-7</td>
<td>Lolium perenne</td>
<td>P. coronata</td>
<td>P. coronata</td>
</tr>
<tr>
<td>NJCC-31</td>
<td>Lolium perenne</td>
<td>P. coronata</td>
<td>P. coronata</td>
</tr>
<tr>
<td>NJCC-33</td>
<td>Lolium perenne</td>
<td>P. coronata</td>
<td>P. coronata</td>
</tr>
<tr>
<td>UK-3</td>
<td>Lolium perenne</td>
<td>P. coronata</td>
<td>P. coronata</td>
</tr>
<tr>
<td>UKF-13</td>
<td>Lolium perenne</td>
<td>P. coronata</td>
<td>P. coronata</td>
</tr>
<tr>
<td>UKF-14</td>
<td>Lolium perenne</td>
<td>P. coronata</td>
<td>P. coronata</td>
</tr>
<tr>
<td>NJNB-37</td>
<td>Poa pratensis</td>
<td>P. coronata</td>
<td>P. striiformis / P. graminis</td>
</tr>
<tr>
<td>NJCC-42</td>
<td>Poa pratensis</td>
<td>P. coronata</td>
<td>P. striiformis / P. graminis</td>
</tr>
<tr>
<td>NJE-50</td>
<td>Poa pratensis</td>
<td>P. coronata</td>
<td>P. striiformis / P. graminis</td>
</tr>
<tr>
<td>MEG-1</td>
<td>Poa pratensis</td>
<td>P. coronata</td>
<td>P. striiformis / P. graminis</td>
</tr>
<tr>
<td>WY-1</td>
<td>Poa pratensis</td>
<td>P. coronata</td>
<td>P. striiformis / P. graminis</td>
</tr>
<tr>
<td>NC-2</td>
<td>Poa pratensis</td>
<td>P. coronata</td>
<td>P. striiformis / P. graminis</td>
</tr>
<tr>
<td>OH-3</td>
<td>Poa pratensis</td>
<td>P. coronata</td>
<td>P. striiformis / P. graminis</td>
</tr>
<tr>
<td>OH-4</td>
<td>Poa pratensis</td>
<td>P. coronata</td>
<td>P. striiformis / P. graminis</td>
</tr>
<tr>
<td>OH-5</td>
<td>Poa pratensis</td>
<td>P. coronata</td>
<td>P. striiformis / P. graminis</td>
</tr>
<tr>
<td>MA-4</td>
<td>Poa pratensis</td>
<td>P. coronata</td>
<td>P. striiformis / P. graminis</td>
</tr>
<tr>
<td>MA-5</td>
<td>Poa pratensis</td>
<td>P. coronata</td>
<td>P. striiformis / P. graminis</td>
</tr>
<tr>
<td>NJA-19</td>
<td>Poa pratensis</td>
<td>P. coronata</td>
<td>P. striiformis / P. graminis</td>
</tr>
<tr>
<td>NJA-20</td>
<td>Poa pratensis</td>
<td>P. coronata</td>
<td>P. striiformis / P. graminis</td>
</tr>
<tr>
<td>NJA-21</td>
<td>Poa pratensis</td>
<td>P. coronata</td>
<td>P. striiformis / P. graminis</td>
</tr>
<tr>
<td>NJA-23</td>
<td>Poa pratensis</td>
<td>P. coronata</td>
<td>P. striiformis / P. graminis</td>
</tr>
<tr>
<td>NIC-28</td>
<td>Poa pratensis</td>
<td>P. coronata</td>
<td>P. striiformis / P. graminis</td>
</tr>
<tr>
<td>NIC-29</td>
<td>Poa pratensis</td>
<td>P. coronata</td>
<td>P. striiformis / P. graminis</td>
</tr>
<tr>
<td>NJHP-32</td>
<td>Poa pratensis</td>
<td>P. coronata</td>
<td>P. striiformis / P. graminis</td>
</tr>
<tr>
<td>NJT-4</td>
<td>Poa pratensis</td>
<td>P. coronata</td>
<td>P. striiformis / P. graminis</td>
</tr>
<tr>
<td>OH-1B</td>
<td>Poa pratensis</td>
<td>P. coronata / P. striiformis</td>
<td>P. striiformis / P. graminis</td>
</tr>
<tr>
<td>OH-8B</td>
<td>Poa pratensis</td>
<td>P. coronata / P. striiformis</td>
<td>P. striiformis / P. graminis</td>
</tr>
<tr>
<td>NIC-8</td>
<td>Zoysia sp.</td>
<td>P. coronata</td>
<td>P. coronata</td>
</tr>
<tr>
<td>NIF-45</td>
<td>Festuca arundinacea</td>
<td>P. graminis</td>
<td>P. graminis</td>
</tr>
<tr>
<td>Host Species</td>
<td>Morphological Characteristics&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Probable Field I.D.&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ITS Sequence Data I.D.</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------</td>
<td>-------------------------------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>NJH-48</td>
<td>Festuca arundinacea</td>
<td>P. graminis</td>
<td>P. graminis</td>
</tr>
<tr>
<td>NJH-49</td>
<td>Festuca arundinacea</td>
<td>P. graminis</td>
<td>P. graminis</td>
</tr>
<tr>
<td>NJT-5</td>
<td>Poa pratensis</td>
<td>P. graminis</td>
<td>P. graminis</td>
</tr>
<tr>
<td>NYPI-1</td>
<td>Poa pratensis</td>
<td>P. graminis</td>
<td>P. graminis</td>
</tr>
<tr>
<td>NJGM-39</td>
<td>Poa pratensis</td>
<td>P. graminis</td>
<td>P. graminis</td>
</tr>
<tr>
<td>ONCA-1</td>
<td>Poa pratensis</td>
<td>P. graminis</td>
<td>P. graminis</td>
</tr>
<tr>
<td>NE-1</td>
<td>Poa pratensis</td>
<td>P. graminis</td>
<td>P. graminis</td>
</tr>
<tr>
<td>NC-1</td>
<td>Poa pratensis</td>
<td>P. graminis</td>
<td>P. graminis</td>
</tr>
<tr>
<td>OH-6</td>
<td>Poa pratensis</td>
<td>P. graminis</td>
<td>P. graminis</td>
</tr>
<tr>
<td>OH-7</td>
<td>Poa pratensis</td>
<td>P. graminis</td>
<td>P. graminis</td>
</tr>
<tr>
<td>MNM-1</td>
<td>Poa pratensis</td>
<td>P. graminis</td>
<td>P. graminis</td>
</tr>
<tr>
<td>NIA-53</td>
<td>Poa pratensis</td>
<td>P. graminis</td>
<td>P. graminis</td>
</tr>
<tr>
<td>NIA-54</td>
<td>Poa pratensis</td>
<td>P. graminis</td>
<td>P. graminis</td>
</tr>
<tr>
<td>NIA-17</td>
<td>Poa pratensis</td>
<td>P. graminis</td>
<td>P. graminis</td>
</tr>
<tr>
<td>NIA-18</td>
<td>Poa pratensis</td>
<td>P. graminis</td>
<td>P. graminis</td>
</tr>
<tr>
<td>OH-1A</td>
<td>Poa pratensis</td>
<td>P. graminis</td>
<td>P. graminis</td>
</tr>
<tr>
<td>OH-8A</td>
<td>Poa pratensis</td>
<td>P. graminis</td>
<td>P. graminis</td>
</tr>
<tr>
<td>PA-2</td>
<td>Lolium perenne</td>
<td>P. coronata / P. striiformis</td>
<td>P. coronata</td>
</tr>
<tr>
<td>NYH-4</td>
<td>Lolium perenne</td>
<td>P. coronata / P. striiformis</td>
<td>P. striiformis</td>
</tr>
<tr>
<td>AU-1</td>
<td>Poa annua</td>
<td>P. striiformis</td>
<td>P. striiformis</td>
</tr>
</tbody>
</table>

<sup>a</sup>Morphological identifications after Cummins (18): Urediniospores (n = 50) of P. coronata = spores 19-25 x 17-21µ, mostly ellipsoid or broadly ellipsoidal, wall 1.5-2µ thick; P. graminis = spores 26-40µ x 16-22µ, globoïd or more or less oblong, wall 1-1.5µ thick at sides, 5-9µ thick apically, equatorially aligned germ pores; and P. striiformis = spores 25-30µ x 20-24µ, mostly broadly ellipsoidal or broadly obovoid, wall 1.5-2µ thick. Teliospores were only present for sample UKF-8 and MA-3 and exhibited 2-celled teliospores with apical digitations, characteristic of P. coronata.

<sup>b</sup>Probable Field I.D. = the likely rust species identification in the field using a combination of pustule phenotype and host plant association. Phenotype identifications after Cummins (18), Smiley et al. (40), Vargas(44): P. coronata = pustules brownish yellow to yellow; P. graminis = pustules cinnamon brown to brown; and P. striiformis = pustules bright orange, in linear streaks along host vein. Host Plant Association after Smiley et al. (40) and Vargas (44): P. coronata = Lolium sp. and Festuca sp.; P. graminis = Lolium sp. and P. pratensis; P. striiformis = Festuca sp. and Poa sp

<sup>c</sup>Multiple species in a column represent inconclusive identification; exact species identification using stated technique resulted in multiple tentative rust species.
Figure 1. Map depicting rust sampling locations in the United States. Darkened states represent locations from which samples were obtained.
Figure 2. Maximum clade credibility Bayesian phylogenetic tree of the three *Puccinia* sp. identified on turfgrass hosts in this study; *P. coronata*, *P. graminis*, and *P. striiformis*. *P. coronata* complex is expanded in Figure 3. 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. A complete list of all taxa is presented in Table 1.
Figure 3. Maximum clade credibility Bayesian phylogenetic tree of *P. coronata* complex 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. A complete list of all taxa is presented in Table 1.
Figure 4. Alignment diagram of the partial ITS 1 region showing the placement of real-time primers FrITS1Pu and RrITS1Pu with *P. 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. Species-specific SNPs differentiating species are denoted by grey 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.
Figure 5. Alignment diagram of the ITS 2 region showing the placement of real-time primers FrITS2Cr and RrITS2Cr with *P. coronata* probe PuCr-ITS2. Representative samples from *P. coronata*, *P. graminis*, and *P. striiformis* on turfgrass and cereal crop hosts are included. Species-specific SNPs differentiating species are denoted by grey and white shading. Probe binding site differed between the three turfgrass rust fungi by at least 5 SNPs. Numbers next to species names represent nucleotide base pair positions.
Figure 6. 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 Ct value (right). A,B). *P. coronata* probe PuCr-ITS2 with lower detection limit of 9pg, C,D). *P. graminis* probe PuSTM-ITS1 with lower detection limit of 1pg, and E,F). *P. striiformis* probe PuSTR-ITS1 with lowest detection limit of 4pg.
Figure 7. Real-time polymerase chain reaction data with *P. graminis* probe PuSTM-ITS1 and *P. striiformis* probe PuSTR-ITS1 multiplexed in a single reaction tube using turfgrass samples. Only target of interest produced fluorescence in each multiplexed reaction. Positive control pUC57 samples are labeled. A). Fluorescence for the FAM labeled probe PuSTR-ITS1 and B). Fluorescence for the Cy3 labeled probe PuSTM-ITS1.
Figure 8. Real-time PCR amplification plot of turfgrass rust probes against a 96-well plate of turfgrass rust isolates. Positive controls synthesized in the pUC57 plasmid vector are denoted by the black arrow. A). Crown rust probe PuCR-ITS2, B). Stem rust probe PuSTM-ITS1, C). Stripe rust probe PuSTR-ITS1
CHAPTER 2. Development of a Greenhouse Based Inoculation Protocol for Isolates of *Puccinia coronata* and *P. graminis* Pathogenic to Kentucky Bluegrass

ABSTRACT

Rust (*Puccinia* sp.) is an aesthetically unappealing fungal disease of Kentucky bluegrass that can cause severe thinning in heavily diseased areas. Resistant cultivars are the primary method of control, and turfgrass breeders have been working to incorporate resistance for a number of years. Successful turfgrass rust inoculation protocols are lacking, along with adequate understanding of pathogen biology, thus delaying the germplasm screening process and our ability to effectively manage rust disease. In this study, we attempted to develop a reliable inoculation protocol for the three most common turfgrass rust species observed in temperate regions - *Puccinia coronata*, *P. graminis*, and *P. striiformis*. Using a modified inoculation protocol adapted from the cereal rust system, uredinia of *P. coronata* and *P. graminis* developed on inoculated Kentucky bluegrass cultivars Bewitched, Diva, and Ulysses approximately 10-14 following inoculation. Morphological evaluations of uredinia pustules were consistent with each respective species, and real-time PCR experiments confirmed the presence of these two species on the inoculated host. However, even after repeat attempts, *P. striiformis* infection never developed. Difficulty maintaining adequate low temperatures for urediniospore germination and infection was likely the limiting factor in the *P. striiformis* experiments, thus additional research in turfgrass hosts is necessary to determine the exact environmental settings necessary. The development of a successful inoculation
protocol for *P. coronata* and *P. graminis* in Kentucky bluegrass will advance turfgrass germplasm screening procedures, as well as allow for the cultivation of homogenous isolates of turfgrass rust fungi for use in future molecular analyses.
INTRODUCTION

Rust (*Puccinia* sp.) is a common fungal disease of Kentucky bluegrass (*Poa pratensis* L.) that has been increasing in frequency in recent years in the northeastern United States (Bonos et al., 2006). Although fungicides can be used to control the disease, the preferred method of control on landscape turf is through the use of resistant cultivars. Efforts to introduce rust resistance into Kentucky bluegrass have been undertaken since the early 1950s, when it became apparent that the leaf spot resistant cultivar, Merion, was highly susceptible to *Puccinia graminis*, the causal agent of stem rust disease (Ray, 1953). It is now common practice for turfgrass breeding programs to evaluate new germplasm for susceptibility to rust disease in the field (Bonos et al., 2006); however, advances in rust resistance in turfgrass is still hindered by the inability of breeders to evaluate turfgrass cultivars in the greenhouse or growth chambers under controlled environments. Additionally, fundamental knowledge of the biology and infectivity of the causal agents is lacking, impeding breeding for resistance against multiple fungal races or strains.

Two primary factors have contributed to the limited advancements in the control and study of turfgrass rust fungi; (1) the inability to obtain pure *in vitro* cultures for pathogen research; and (2) difficulty in developing a reliable inoculation protocol for evaluating plants in the growth chamber or greenhouse. The obligate nature of rust fungi makes them extremely difficult, if not impossible, to grow in culture (Agrios, 2005). Attempts to grow graminicolous rust fungi in the laboratory have proven to be complicated, and frequently produce non-repeatable results (Huang et al., 1990, Williams
Detached leaf assays have been used to maintain cultures of *P. coronata* from oats (*Avena sativa* L.), however, it remains to be determined if this technique can be used to screen healthy plants for resistance to rust (Jackson et al., 2008), as leaf senescence may be problematic.

The complexities surrounding axenic culturing of graminicolous rust fungi has been overcome in the cereal rust system, where inoculation of cool-season cereal crops, including wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.) and oats, is a routine procedure that has been performed for several decades (Jackson et al., 2008). In this system, viable urediniospores are sprayed onto susceptible plants in a mineral oil suspension and placed in a dew chamber overnight (Carson, 2008). The following day, inoculated plants are moved to the greenhouse where uredinia are allowed to develop, a process that takes about ten days (Carson, 2008). When pustules erupt, a single uredinium is selected and allowed to expand (Carson, 2008). Ultimately, spores from the single pustule are harvested and used to inoculate fresh plant material, thus increasing the inoculum of a single isolate (Carson, 2008). This method ensures pathogen homogeneity and produces copious amounts of genetically identical spores that can then be used for advanced molecular analyses or to screen germplasm.

A similar procedure has been effectively adapted to other grass systems, such as in Italian ryegrass (*Lolium multiflorum* Lam.), where inoculations of *P. coronata* were used to understand resistance markers in the host plant (Studer et al., 2007). The successful inoculation of Kentucky bluegrass, perennial ryegrass (*Lolium perenne* L.), and tall fescue (*Festuca arundinacea* Schreb.) with isolates of *P. graminis* has also been
described (Pfender 2009, 2003, 2001), offering promise that this procedure may be used with other species of turfgrass rust fungi.

In addition to *P. graminis*, at least two other *Puccinia* species, *P. coronata* (Beirn et al., 2011, see chapter 1) and *P. striiformis* (Smiley et al. 2005), are known to infect Kentucky bluegrass in temperate regions, both of which are also important targets of turfgrass breeding programs (Bonos et al., 2006). Despite the obvious benefits a successful, reproducible inoculation procedure would provide breeders and pathologists, a universal inoculation protocol has not been developed for *P. coronata*, *P. graminis*, and *P. striiformis* from turf, although attempts have been made by some laboratories (S.A. Bonos, personal communication; E. Watkins, personal communication). The development of an effective inoculation protocol for turfgrass rust fungi would allow researchers to genetically purify rust strains for pathogen analysis at the molecular level, and provide the ability to screen large numbers of turfgrass selections and cultivars in the greenhouse for resistance to known species/races in a convenient, reproducible manner before planting. Therefore, our objective in this study was to develop a reliable inoculation protocol for turfgrass rust fungi by adapting and modifying techniques developed and used in the cereal rust system.
MATERIALS AND METHODS

Turfgrass hosts

Kentucky bluegrass cultivars Bellisimo, Bewitched, Diva, A98-708, and Ulysses were seeded in 10 cm pots filled with Fafard Canadian Grow Mix 2 (Agawam, MA). A98-708 is an experimental cross between the two popular cultivars Eagleton and Midnight. These cultivars were selected based on their observed susceptibility to rust diseases in turfgrass research plots at the Rutgers University Research Farm in Adelphia, New Jersey (W.A. Meyer, personal communication). To control outbreaks of powdery mildew [Blumeria graminis (DC.) E.O. Speer] during seedling establishment, elemental sulfur was burned in a sulfur vaporizer (Better Grow Hydro, Bell, CA) overnight, for a period of 8 h. Two sulfur treatments were applied before rust inoculations; one treatment on 1 wk old plants followed by a treatment on 2 wk old plants.

Fungal samples

Isolates of Puccinia coronata, P. graminis, and P. striiformis were collected in the summer of 2009 and 2010. Prior to inoculation experiments, urediniospores were removed from -70°C storage, where they were maintained as desiccated urediniospores in 50 mL polypropylene tubes (BD Falcon, Bedford, MA), and were allowed to acclimate to room temperature for 1-2 h. Urediniospore germination was tested by streaking spores on 2% water agar and incubating the plates on the laboratory bench top for several hours; only samples with ≥80% germination were used for inoculation. One isolate of P.
*coronata, P. graminis, and P. striiformis* with sufficient germination was used for these studies (Table 1).

**Inoculation procedure**

All cultivars were inoculated with each of the three *Puccinia* species. The inoculation protocol consisted of 0.10 g of urediniospores suspended in 1 mL of light mineral oil (Fisher Scientific, Pittsburgh, PA) in a 1.5mL microcentrifuge tube and mixed by vortexing for 10 seconds. The mineral oil/spore suspension was sprayed onto the foliage of 5 wk post-emergent Kentucky bluegrass plants in a fume hood using a Badger 150 airbrush paint sprayer (Franklin Park, IL) with the bottom feed head adapter. Non-inoculated Kentucky bluegrass plants were sprayed with water and served as a control for all experiments. Inoculated plants were allowed to dry for approximately 1 h in the fume hood, and were then transferred to a custom-made plexi-glass misting chamber (Figure 1). The plexi-glass chamber measured 91.5 cm x 63.5 cm x 76.2 cm and was constructed with 7.9 mm plexi-glass. A humidifier (Herrmidifier, Phoenix, AZ) provided the source of mist in the chamber. Inoculated plants were incubated in the misting chamber for 10 h overnight, with misting/dry cycles alternating every 30 minutes. The air temperature inside the misting chamber fluctuated between 18 to 20°C. For *P. graminis* inoculations, the misting chamber was covered with a dark cloth during the first two hours of incubation to ensure a sufficient overnight dark period was met (Pfender, 2001). After 10 h, the misting chamber door was opened and plants were allowed to acclimate to the greenhouse for 1-2 h. Inoculated plants were then transferred to custom-built wood-framed incubation chamber cubes, measuring 30.5 cm x 30.5 cm on all sides. Three of
the six sides of the incubation chambers were covered with 4 mm translucent plastic (Film-Gard, Inc.) to keep plants separated, while still allowing exposure to light (Figure 2). Plants inoculated with *P. coronata* or *P. graminis* spores were kept in the wood-framed bench top inoculation chamber cubes in the greenhouse until pustules developed. Day-time temperatures were maintained between 19 to 21°C beginning 2 h after dawn and ending 1 h before dusk. Night temperatures were maintained between 18 to 20°C. The preset light intensity conditions were as follows: overhead halide lighting illuminated the plants between the hours of 7 am and 7 pm, only if the light intensity dropped below 350 µE m$^{-2}$s$^{-1}$. If the light intensity exceeded 900 µE m$^{-2}$s$^{-1}$, 50% of the greenhouse was shaded with shade cloth to reduce light levels. If the light intensity reached >1200 µE m$^{-2}$s$^{-1}$, 95% of the greenhouse was shaded by shade cloth to reduce light levels.

Because *P. striiformis* prefers cooler temperatures (Smiley et al., 2005), plants inoculated with *P. striiformis* were placed in inoculation chambers in a 12°C growth chamber, under 30-40 % RH, and a 12 h photoperiod at intensity 600 µE m$^{-2}$s$^{-1}$. All plants were watered at the base to avoid wetting the inoculated foliage during the course of the experiments.

**Spore Morphology and Real-time PCR**

Upon uredinia development, spores were harvested by hand to confirm species identity. Morphological evaluations consisting of measuring urediniospore size, shape, color, and the number of germ pores were performed using Cummins (1971) as a guide. Germ pores were stained and visualized using the analine-blue squash technique (Jennings et al., 1989) and spores were photographed using an Olympus BX41 clinical
Urediniospore DNA was extracted using the OmniPrep Extraction Kit (G-Biosciences, Maryland Heights, MO) and each sample was screened against real-time PCR probes designed for the detection of *P. coronata*, *P. graminis*, and *P. striiformis* from turfgrass hosts (Beirn et al., 2011, see chapter 1). Real-time PCR reactions were performed using a Cepheid SmartCycler (Cepheid, Sunnyvale, CA) in 25 µl Cepheid tubes. Cepheid’s Smartmix HM lyophilized PCR master mix was used for all reactions with a final probe concentration of 1µM and primer concentration of 10µM. 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.
RESULTS

Inoculation results

Spore solutions of *P. coronata*, *P. graminis*, and *P. striiformis*, were individually inoculated onto 5 wk old post-emergent Kentucky bluegrass cultivars Bellisimo, Bewitched, Diva, A98-708, and Ulysses. Approximately 10-14 days post-inoculation, pustules of *P. coronata* and *P. graminis* were observed on cultivars Bewitched, Diva, and Ulysses (Table 2, Figures 3A, 4A). Control plants never developed rust pustules. Bellisimo and the experimental cultivar A98-708 sustained extreme powdery mildew outbreaks, despite treatments with vaporized sulfur; thus, had no visible rust infections.

*P. coronata* pustules were a bright yellow-orange color, formed on the adaxial leaf surface, and were slightly oblong to round in shape. Uredinia were randomly distributed from mid-leaf to the leaf tips of inoculated cultivars, reflecting the points of inoculation. Infection developed on approximately 50% of the total pot surface area for the cultivars Bewitched and Diva, while Ulysses exhibited about 30% infection. No infection was seen on leaf sheaths or plant crowns on any cultivars. Plants appeared healthy and no chlorotic or necrotic lesions were present.

*P. graminis* uredinia were dark red in color, located on the adaxial leaf surfaces, and were oblong in shape. Uredinia were only located mid-leaf, with no infection on the leaf sheaths or crowns. All three cultivars exhibited about 30% infection on the total pot surface area, which was typically located on leaves exposed to the pot perimeter. No chlorotic or necrotic lesions were present. The observed symptoms were consistent with reports by Cummins (1971) and Smiley et al. (2005).
Photographs of *P. coronata* and *P. graminis* uredinia and urediniospores are present in Figures 3 and 4. Urediniospores harvested from the infected plants were evaluated for spore size, shape, color, and number of germ pores. *P. coronata* urediniospores were approximately 20-25 x 17-22µ, mostly ellipsoid, yellowish to orange, and had 4-6 scattered germ pores (Figures 3B-D) which is consistent with descriptions of the fungus (Cummins, 1971) and the isolate used to inoculate the plants. *P. graminis* urediniospores were 26-39µ x 16-23µ, globoid or oblong, dark red to brown, and had 3 equatorial germ pores (Figures 4B-D) and was consistent with descriptions of the fungus (Cummins, 1971) and the isolate used to inoculate the plants.

Because of the powdery mildew infections experienced in the *P. graminis* and *P. coronata* experiments, the cultivars Bellisimo and A98-708 were eliminated from *P. striiformis* experiments. Plants inoculated with *P. striiformis* urediniospores never developed disease symptoms, even after repeated attempts.

**Real-time PCR confirmation**

Species identification was confirmed by using the real-time PCR probes previously developed for the identification of turfgrass rust fungi (Chapter 1; Beirn et al., 2011, see chapter 1). *P. graminis* and *P. coronata* were detected from all inoculated Kentucky bluegrass plants using real-time PCR probes, with average C_T values of 26.88 and 28.79, respectively (Figure 5A and B). *P. striiformis* was not detected by the *P. striiformis* probe (data not shown) when used to evaluate the *P. striiformis* inoculated plant tissue.
DISCUSSION

The primary objective of this study was to develop a reliable inoculation protocol for the three species of turfgrass rust fungi commonly observed on Kentucky bluegrass in temperate regions—*P. coronata*, *P. graminis*, and *P. striiformis* (Beirn et al., 2011, see chapter 1, Smiley et al., 2005). Three Kentucky bluegrass cultivars were successfully inoculated with both *P. coronata* and *P. graminis*, while *P. striiformis* infection failed to develop on any of the inoculated cultivars. Italian ryegrass (Studer et al., 2007) and Kentucky bluegrass (Pfender et al. 2009) have been successfully inoculated with *P. coronata* and *P. graminis*, respectively; however, the protocol implemented in the current study is the first universal inoculation procedure developed for both rust species on Kentucky bluegrass.

While we were unable to observe infection with *P. striiformis* in Kentucky bluegrass, effective inoculations have been performed in wheat (Chen et al., 2009, de Vallavieille-Pope et al., 1995, Duan et al., 2010). In this system, *P. striiformis* f. sp. *tritici* urediniospore germination was heavily dependent on temperature, light intensity, and the duration of wet periods. At low light intensities (150-300 µE m$^{-2}$s$^{-1}$), susceptible wheat cultivars exhibited almost complete resistance to *P. striiformis* f. sp. *tritici* infection when compared to inoculations at a light intensity of 600 µE m$^{-2}$s$^{-1}$ (Ash and Rees, 1994). Similarly, interruption of the wet period for only one hour resulted in death of *P. striiformis* f. sp. *tritici* appressoria (de Vallavieille-Pope et al., 1995). Maximum urediniospore germination has been observed between 8 and 12°C. Although 5 to 18°C is considered acceptable (de Vallavieille-Pope et al., 1995), no germination has been
reported above 20°C (Ash and Rees, 1994, de Vallavieille-Pope et al., 1995). In the current turfgrass rust inoculation protocol, light intensity remained stable throughout the experiment at 600 µE m⁻² s⁻¹, thus temperature and leaf wetness may have been the limiting factors for *P. striiformis* infection in Kentucky bluegrass. The temperatures within the growth chambers routinely exceeded 12°C and the lack of a constant misting source as in the *P. coronata/P. graminis* misting chamber may have interrupted urediniospore germination, penetration, and infection.

The success demonstrated here with *P. coronata* and *P. graminis* inoculations indicates that a similar inoculation process can be developed for isolates of *P. striiformis* from turfgrass, though equipment limitations must be overcome and additional research needs to be conducted to determine the specific parameters needed to successfully generate artificial infection for this pathogen on turf. Additionally, it cannot be ruled out that the Kentucky bluegrass cultivars used in our study were not highly susceptible to the isolate of *P. striiformis* used for inoculation; therefore additional cultivars and fungal isolates shall be screened and inoculated in the future.

Despite the difficulty initiating *P. striiformis* infection, development of a successful *P. coronata* and *P. graminis* rust inoculation protocol for Kentucky bluegrass marks a significant advance and should assist in developing a better understanding of turfgrass rust fungi in the future. Researchers now have the ability to genetically purify isolates of *P. coronata* and *P. graminis*, increase inoculum, and conduct future studies derived from single-spore isolates. Moreover, turfgrass breeders now have a reliable method for screening Kentucky bluegrass cultivars in the greenhouse or growth chamber to assess resistance to known species and/or races of rust prior to planting in the field,
significantly reducing the time and resources required to develop rust resistant turfgrass
cultivars. This procedure may also be used as a foundation for establishing a
standardized set of race differentials in Kentucky bluegrass; a regular practice used in the
cereal rust system to determine the emergence and severity of new rust races against a
series of known host plant resistance genes (Roelfs, 1984).
REFERENCES


Table 1. Fungal isolates used for the greenhouse-based inoculation protocol. Urediniospores were collected from field plots comprised of multiple cultivars.

<table>
<thead>
<tr>
<th>Species</th>
<th>Isolate</th>
<th>Host Plant</th>
<th>Location</th>
<th>Date Collected</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Puccinia coronata</em></td>
<td>CRV-A1</td>
<td><em>Poa pratensis</em></td>
<td>Adelphia, NJ</td>
<td>00-Jun-2009</td>
</tr>
<tr>
<td><em>Puccinia graminis</em></td>
<td>SRV-A2</td>
<td><em>Poa pratensis</em></td>
<td>Adelphia, NJ</td>
<td>00-Jun-2009</td>
</tr>
<tr>
<td><em>Puccinia striiformis</em></td>
<td>STR-HF2</td>
<td><em>Poa pratensis</em></td>
<td>North Brunswick, NJ</td>
<td>00-Dec-2009</td>
</tr>
</tbody>
</table>
Table 2. Results of inoculation experiments using different Kentucky bluegrass cultivars and fungal isolates of *Puccinia coronata*, *P. graminis*, and *P. striiformis*, where + = infection was present and - = no infection.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th><em>Puccinia coronata</em> CRV-A1</th>
<th><em>Puccinia graminis</em> SRV-A2</th>
<th><em>Puccinia striiformis</em> STR-HF2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bellisimo</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bewitched</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Diva</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>A98-708</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ulysses</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 3. Morphological and real-time PCR evaluations of harvested *Puccinia coronata* and *P. graminis* urediniospores from three inoculated Kentucky bluegrass cultivars.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Bewitched</td>
<td><em>P. coronata</em></td>
<td><em>P. coronata</em></td>
<td><em>P. graminis</em></td>
<td><em>P. graminis</em></td>
</tr>
<tr>
<td>Diva</td>
<td><em>P. coronata</em></td>
<td><em>P. coronata</em></td>
<td><em>P. graminis</em></td>
<td><em>P. graminis</em></td>
</tr>
<tr>
<td>Ulysses</td>
<td><em>P. coronata</em></td>
<td><em>P. coronata</em></td>
<td><em>P. graminis</em></td>
<td><em>P. graminis</em></td>
</tr>
</tbody>
</table>
Figure 1. Photograph depicting the plexi-glass misting chamber used to incubate Kentucky bluegrass plants previously inoculated with *Puccinia* species. A hole was cut though the back wall to allow insertion of a humidifier, which provided the misting source.
Figure 2. Photograph depicting the custom-built wood framed inoculation chambers.
Figure 3. A. *Puccinia coronata* uredinia on inoculated Kentucky bluegrass, B. *P. coronata* urediniospores (400x); C. a germinating urediniospore (400x) of *P. coronata*; and D. a squashed *P. coronata* urediniospore with visible, scattered germ pores (400x).
Figure 4. A. *Puccinia graminis* uredinial sori erupting from inoculated Kentucky bluegrass; B. *P. graminis* urediniospores (100x); C. *P. graminis* urediniospores (400x); and D. a squashed *P. graminis* urediniospore with visible, equatorial germ pores (400x).
Figure 5. Real-time PCR data from inoculation experiments. Only the target of interest produced fluorescence. A. Fluorescence of *Puccinia coronata* isolate CRV-A1 from inoculated *P. pratensis* cultivars Bewitched, Diva, and Ulysses. B. Fluorescence of *P. graminis* isolates SRV-A2 from inoculated *P. pratensis* cultivars Bewitched, Diva, and Ulysses.
Curriculum Vitae

LISA ANNE BEIRN

Education:

September 2008-present  Rutgers, The State University of New Jersey
Master's Student in Plant Biology

September 2003-May 2007  Rutgers, The State University of New Jersey
Bachelor's of Science in Biological Sciences, with honors

Occupations:

September 2008-present  Graduate Research Assistant, Rutgers University,
Dept. of Plant Biology

May 2006-August 2008  Laboratory Technician, Plant Diagnostic Lab,
Rutgers University

Publications:

rusts reveals the widespread distribution of Puccinia
coronata as a pathogen of Kentucky bluegrass in the U.S.
Plant Disease: PDIS-01-11-0073-RE.

Clarke, B.B. 2009. Anthracnose disease of switchgrass
caused by the novel fungal species Colletotrichum navitas,
Mycological Research (113): 1411-21.

and grasses. Fungal Diversity (39): 19-44.