MOLECULAR INSIGHTS INTO THE MICROBIAL COMMUNITY OF ANNUAL BLUEGRASS (*POA ANNUA* L.) PUTTING GREEN TURF

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ABSTRACT OF THE DISSERTATION

Molecular Insights into the Microbial Community of Annual Bluegrass (*Poa annua* L.) Putting Green Turf

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Annual bluegrass (*Poa annua*; ABG) putting green turf is a unique man-made environment that requires regular fertility inputs to maintain acceptable turfgrass quality and playability. While these inputs can affect foliar diseases such as anthracnose, caused by *Colletotrichum cereale*, little is known about their impact on microbial communities in this ecosystem. The objectives of this dissertation were to: 1) determine the frequency and distribution of *C. cereale* in the United States, 2) examine the resident microbial communities in the soil of ABG putting green turf over time using advanced molecular technologies, and 3) identify how nitrogen (N) and/or potassium (K) fertilization affects the distribution, diversity, and abundance of benign and pathogenic microorganisms in this system. More than 50 phyla, representing hundreds of species of archaea, bacteria, and fungi were identified. Above ground, this diversity was highlighted in the form of two distinct lineages of *C. cereale*, both able to cause anthracnose disease but exhibiting distinct host and geographic preferences. Below ground, the ABG rhizosphere supported a vast microbial community, despite high sand content and regular fertilization and pesticide applications. Few turfgrass pathogens were identified from the soil. However,

tremendous variation was characterized within the nonpathogenic microbial community, with the rhizosphere of ABG hosting organisms capable of antibiotic production, fixing nitrogen, or serving as potential biocontrol agents or mycorrhizal partners. Over all, individual microbial groups were present in low abundance across all samples. Fertilization did not affect microbial diversity, but did alter the abundance of specific microbial groups. Changes associated with fertility treatments were limited to approximately 7% of the total archaea/bacteria and 23% of the total fungal community identified. In general, K and low rates of N increased abundance of archaea, bacteria, and fungi in the study sites. Seasonality also strongly influenced microbial communities, with samples collected in summer months clustering separately from those obtained in the spring. The research described here provides the first insight into the diverse microbial community residing in the soil of ABG putting green turf utilizing next-generation sequence-based analyses, and protocols developed to conduct this work should help facilitate future research examining the turfgrass microbiome.

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LITERATURE REVIEW

The Value and Benefits of Turfgrass: A Brief Introduction

Humans have utilized turfgrass for centuries. One of the earliest reports of turf as a recreational surface dates back to the thirteenth century, where turf was used for a sport similar to lawn bowling (Beard 1973). Today, turfgrasses are used in home lawns, athletic fields, golf courses, roadsides, cemeteries, parks and commercial properties. In the United States alone, the turfgrass industry is estimated at $40 billion (National Turfgrass Federation 2009), an income that in some states is larger than from any other agricultural commodity (Breuninger et al. 2013). The total turfgrass area in the United States is estimated to encompass at least 20.2 million hectares (50 million acres) (National Turfgrass Federation 2009), with home lawns and roadsides the dominant locations (Breuninger et al. 2013). Turf utilized for other purposes typically represents only a small percentage (no more than 5%) of the total area covered by turfgrass in any state (Breuninger et al. 2013).

Turfgrasses provide many benefits. Mowed lawns offer a prime location for recreational and relaxation activities, promoting physical wellness and mental well-being (Stier et al. 2013). From an environmental standpoint, turfgrass can assist in erosion control and help recharge groundwater (Beard and Green 1994). In urban areas, where fossil fuel emissions can be present in high concentrations, turfgrass can reduce atmospheric pollutants (Stier et al. 2013) and aid in noise abatement. Turf can also have a cooling effect in these environments due to evapotranspiration, thereby reducing energy consumption (Stier et al. 2013). As a result, managed turfgrass has become an important component of many landscapes.

Putting Greens in the Northeastern U.S.

In the northeastern U.S., two grasses are most frequently utilized for putting greens: creeping bentgrass (*Agrostis stolonifera* L.) and annual bluegrass (*Poa annua* L.). Annual bluegrass is generally regarded as a weed species in the landscape due to its invasive nature and low tolerance of environmental stresses (Turgeon 2005); however, it is frequently found as a main component of golf course putting greens in the northeastern U.S. due to its ability to invade established turfs and persist under low mowing height (Mao and Huff 2012).

Annual bluegrass has two forms: *P. annua* L. f. *annua* Timm., exhibiting an annual growth habit, and *P. annua* L. f. *reptans* (Hausskn.) T. Koyama that exhibits a more perennial form (Mao and Huff 2012). The latter has become quite prevalent on golf course putting greens in the northeastern U.S. because of its prostrate growth habit and its ability to tolerate low mowing height (Huff 2003). In a putting green environment, annual bluegrass is susceptible to a number of fungal diseases, such as dollar spot [*Sclerotinia homoeocarpa* (F.T. Bennett), summer patch (*Magnaporthiopsis poae* (Landsch. & N. Jacks.) J. Luo & N. Zhang), brown patch (*Rhizoctonia solani* Kühn), Pythium blight (*Pythium sp.* Pringsh.) and anthracnose (*Colletotrichum cereale* Manns sensu lato Crouch, Clarke, and Hillman) (Smiley et al. 2005).

In the past 15 years, anthracnose disease has become the most common and destructive disease on annual bluegrass putting greens, with widespread epidemics being reported in North America, the United Kingdom, and Europe (Murphy et al. 2008, Mann and Newell 2005). It has been hypothesized that this increase in disease incidence and severity have been the result of stressful management practices used on golf course

putting greens to improve playability of the turf (Vermeulen 2003, Zontek 2004). Increased playability is often associated with a faster green speed, also known as ball roll distance. To meet the demands for faster green speeds, turfgrass managers have often reduced the mowing height, decreased irrigation rates, and reduced nitrogen (N) fertility on greens (Vermeulen 2003, Zontek 2004). Due to the intensive nature of management programs and the observed increase of anthracnose disease on annual bluegrass putting greens maintained under these regimes, a number of studies were initiated to determine the effects of these rigorous management practices on anthracnose disease development (Hempfling et al. 2011, 2014, 2015, Inguagiato et al. 2008, 2009a, 2009b, 2010, 2012, Murphy et al. 2008, 2012, 2013, Roberts et al. 2011, 2012, Schmid et al. 2010, 2011, 2012, 2013, Wang et al. 2012).

***Colletotrichum cereale*: A Common Pathogen of Annual Bluegrass Turf**

Anthracnose disease of turfgrass is caused by the fungus *Colletotrichum cereale* Manns sensu lato Crouch, Clarke, and Hillman (Crouch et al. 2006), and is perhaps the most-studied microorganism found in association with annual bluegrass turf. *Colletotrichum cereale* has had a storied taxonomic history, undergoing several major revisions since the species was first described on Kentucky bluegrass (*Poa pratensis* L.) in 1909 (Selby and Manns). In 1914, several graminicolous *Colletotrichum* species, including *C. cereale*, were placed in the species *C. graminicola* (Ces.) C. G. Wils., based on similarities in host range and morphological characteristics (Wilson 1914). Teasing apart of the large *C. graminicola* group began in the 1960s, when Sutton reestablished *C. caudatum*, causal agent of anthracnose on warm-season grasses, *C. falcatum*, and *C.*

sublineola, causal agent of sorghum anthracnose, as separate species (Sutton 1965, 1966, 1968). Noticing the distinct appressoria and hyphae of the corn anthracnose pathogen, Sutton also restricted *C. graminicola* to include only those isolates that were pathogenic to corn (Sutton 1966). While Sutton recognized that this treatment left many graminicolous *Colletotrichum* species without a proper name, he did not reclassify any additional species associated with grass hosts and the name *C. graminicola* continued to be used.

In 2006, phylogenetic analysis of the internal transcribed spacer (ITS) region, the conserved mating-type locus (MAT1-2), and the single copy manganese-type superoxide dismutase gene (SOD2) of 107 isolates of *C. graminicola* from grasses revealed three well-supported groups (posterior probabilities = 0.99 to 1): a group comprising isolates from corn, a group comprising isolates from sorghum, and a group comprising isolates from Pooid grasses (Crouch et al. 2006). As a result of these findings, *C. graminicola* was restricted to include only isolates from corn, *C. sublineolum* to isolates obtained from sorghum, and *C. cereale* for isolates of anthracnose inhabiting and causing disease of cool-season grasses (Crouch et al. 2006).

Within the *C. cereale* group, the species was further subdivided into two major clades, currently termed clade A and clade B (Crouch et al. 2006). Clade A isolates were found more frequently than clade B isolates; however, clade B isolates were more genetically diverse, with more unique haplotypes observed from fewer isolates (Crouch et al. 2006). There is host-specificity within the clades, with isolates pathogenic to cultivated turfgrass typically forming their own distinct populations separate from isolates obtained from cereal crops and prairie grasses (Crouch et al. 2009). The exact

biological significance of clades A and B remain unknown, but given the great genetic diversity present within the species and the evidence for recombination between the lineages (Crouch et al. 2006, 2009), it is possible that these two groups played a role in the recent increased incidence of anthracnose disease on golf course putting greens in North America.

Symptomology and the Disease Cycle

When conditions are favorable for disease development, *C. cereale* infects turf causing either a foliar blight and/or a basal rot (Smiley et al. 2005). Infection typically occurs in hot, humid weather (29-35°C) (Smith et al 1989, Smiley et al 2005, Murphy et al 2008), although symptoms have been reported over a wider range of temperatures (15-35°C) (Inguagiato et al. 2008). In foliar infections, symptomatic leaves appear yellow or reddish brown, sometimes with oblong, chlorotic leaf lesions, eventually turning necrotic (Smiley et al. 2005, Smith et al. 1989). Basal rot begins as small, 6-12 mm patches that appear slightly yellowish, orange, or reddish-brown in color (Smiley et al. 2005). Infected plants may exhibit both healthy and infected, yellow tillers, with the youngest leaves changing color last (Smiley et al. 2005). As the disease progresses, these small patches often coalesce and die, forming large, irregular patches of dead turf (Smiley et al. 2005). The crowns of infected plants appear black, water soaked, and often lack a substantial root system (Smith et al. 1989, Smiley et al. 2005). In both the foliar and basal rot stages, heavily melanized acervuli with protruding black setae may be found on dead, dying, or asymptomatic tissue (Vargas et al. 2003, Smiley et al. 2005). Conidia form within acervuli, and then are splashed or blown to nearby, healthy plants, thereby repeating the disease cycle (Vargas et al. 2003).

Infection Cycle

Colletotrichum cereale isolates pathogenic to turfgrass have been hypothesized to overwinter in plant debris or the soil (Smiley et al. 2005). Observations in cereal crops support this theory, as the fungus in this system is known to overwinter as sclerotia on rhizomes or as mycelia in cereal crops (Sanford 1935, Caglevic 1960, Selby and Manns 1909). Molecular analysis of *C. cereale* populations from cereal, prairie, and turfgrass hosts show strong differentiation based on ecotype, suggesting that non-turfgrass hosts probably do not harbor *C. cereale* inoculum that would infect turfgrasses, and that outbreaks of anthracnose on golf courses are likely initiated by overwintering inoculum surviving putting greens (Crouch et al. 2009). Transmission may also occur via the movement of spores on equipment or by wind or rain from nearby infected turf (Murphy et al. 2008).

For *C. cereale*, dark, melanized acervuli with up to seven, black colored setae form in necrotic tissue, from which conidia are produced and serve as a source of secondary inoculum (Crouch et al. 2006, Crouch and Beirn 2009). Conidia are typically falcate or fusiform, hyaline (salmon colored in mass), guttulate, and measure 23.3 μm X 34 μm, on average (Crouch et al. 2006). Conidial development is favored when temperatures range from 29-35°C (Danneberger et al. 1984), and is enhanced when exposed to constant light intensity (Crouch et al. 2006). In corn, *C. graminicola* conidia are excreted from acervuli in a matrix of glycoproteins that aid in pathogenicity and survivability (Nicholson and Moraes 1980, Bergstrom and Nicholson 1999), though this remains to be confirmed for *C. cereale.*

Conidia of *C. cereale* begin to germinate once in contact with a susceptible turfgrass host, under conditions that are conducive to infection. Using detached leaf assays of creeping bentgrass and annual bluegrass, this process was documented to occur in as little as two hours post-inoculation (Khan and Hsiang 2003). From germinating conidia, a single, hyaline germ tube is formed, though occasionally two or more are present (Crouch et al. 2006, Khan and Hsiang 2003). In rare instances, no germ tubes are produced; however this does not appear to inhibit infection (Crouch et al. 2006, Khan and Hsiang 2003). Within six hours, dark brown or black appressoria are formed (Crouch et al. 2006, Khan and Hsiang 2003). Appressoria are typically rounded, lobate, or multi-lobate, measuring 8.5 to 11.6 μm x 6.5 to 10.2 μm (Crouch et al. 2006). They are separated from the germ tube by a septum (Khan and Hsiang 2003, Crouch et al. 2006).

At 6 to 8 hours post-inoculation, penetration pores develop in the center of appressoria; however, penetration pegs are difficult to visualize due to their small size and because they are embedded within the cell wall (Khan and Hsiang 2003). From these infection points, single or double infection hyphae are typically observed within the host epidermal cells at 24 hours post-inoculation (Khan and Hsiang 2003). Further detail about the infection process in *C. cereale* is currently unknown; however, in *C. graminicola*, this marks the beginning of a short biotrophic phase, where the fungus grows intracellularly via thick primary hyphae (Mims and Vaillancourt 2002). The primary hyphae continue to colonize several cells, during which the host remains asymptomatic (Mims and Vaillancourt 2002). Transition to the necrotrophic stage occurs shortly thereafter, and is identified by the presence of numerous, thin secondary hyphae that proliferate throughout the host tissue, behind the advancing primary hyphae (Mims

and Vaillancourt 2002). The host tissue is then rapidly degraded and acervuli begin to form in necrotic tissue (Wharton et al. 2001). This unique lifecycle, combining both biotrophy and necrotrophy, is termed hemibiotrophy, and is a common characteristic of many *Colletotrichum* species (Perfect et al. 1999). Evidence suggests that *C. cereale* also employs a hemibiotrophic infection strategy (Khan and Hsiang 2003); however additional experiments need to be designed to test this hypothesis, as detached leaf assays may not reflect *in planta* infection.

Like many other grass infecting *Colletotrichum* species, the teleomorph has not been observed for *C. cereale* (Crouch and Beirn 2009). Characterization of the *Mat1* gene in *C. cereale* has only revealed the presence of the *Mat1-2* idiomorph (Crouch et al. 2006), suggesting that an additional gene or genes located in another region of the genome may regulate sexual reproduction (Vaillancourt et al. 2000). Regardless, research supports the concept that recombination has occurred within *C. cereale* at some point. Reticulating network topologies of *C. cereale* haplotypes have revealed considerable genetic flow between populations of this fungus, particularly within clade B isolates (Crouch et al. 2009). Similarly, estimates of recombination (ϕ_w) were high for four markers sequenced from 208 isolates of *C. cereale* (Crouch et al. 2009). Furthermore, repeat-induced point (RIP) mutations were identified in 21 of 35 transposable elements found in clade B isolates (Crouch et al 2008). RIPs are a common mechanism employed by fungi for silencing transposable elements that occurs solely during meiosis, therefore their presence within the *C. cereale* genome suggests that sexual recombination has occurred at some point in time (Crouch et al. 2008).

Reducing Disease Through the Use of Best Management Practices

Anthracnose disease can be controlled chemically in turfgrass; however, exercising good cultural management practices can suppress disease, reduce fungicide use (Hempfling et al. 2014), and provide a more environmentally friendly approach to disease control (Murphy et al. 2008, Murphy et al. 2012). Initial research indicated that low mowing heights and reduced nitrogen fertilization increased the severity of anthracnose disease on annual bluegrass putting green turf (Inguagiato et al. 2008). Thus, several additional studies were implemented to examine the effects of other frequently used management and cultivation techniques on anthracnose severity. To date, the following management/cultivation practices have been investigated: plant growth regulators (Inguagiato et al. 2008, 2009b, 2010), topdressing (Inguagiato et al. 2012, Hempfling et al. 2015, Wang et al. 2012), irrigation (Roberts et al. 2011), rolling (Inguagiato et al. 2009a, Roberts et al. 2012), verticutting (Inguagiato et al. 2008), and scarification (Hempfling et al. 2011). While all of these practices have been shown to impact anthracnose, nitrogen fertility has been reported to have the greatest effect on the severity of this disease (Inguagiato et al. 2008, Schmid et al. 2010, 2011, 2012).

In the field, research has shown that low rates of N (4.9 kg ha^{-1}) applied every seven days can reduce anthracnose severity between 5 and 24% compared to the same rate applied at 28-day intervals (Inguagiato et al. 2008). However, when extremely high rates were examined (19.6 and 24.5 kg ha^{-1} applied every 7 days), they enhanced anthracnose severity, indicating that too little or excessive N fertility exacerbates anthracnose disease (Schmid et al. 2010).

Nitrogen source also plays a role in the development of anthracnose in turfgrass. In a two-year N source study, Schmid et al. examined the effects of ammonium sulfate ($(NH_4)_2SO_4$), ammonium nitrate (NH_4NO_3), urea (CH_4N_2O), calcium nitrate ($Ca(NO_3)_2$), and potassium nitrate (KNO_3) on disease severity on annual bluegrass putting green turf (2012). Although all N sources reduced disease severity compared to untreated turf, plots fertilized with nitrate sources had the least amount of disease, whereas plots fertilized with ammonium nitrogen had the greatest disease severity (Schmid et al. 2012). More specifically, KNO_3 plots performed the best (best turf quality and least amount of disease), while $(NH_4)_2SO_4$ performed the worst, indicating that soil pH, as well as K, may influence disease severity (Schmid et al. 2012).

To further examine how potassium (K) may affect anthracnose disease development, another field study was initiated in 2012 to investigate the effect of various potassium sources [potassium sulfate (K_2SO_4), potassium chloride (KCl), potassium carbonate (K_2CO_3) and potassium nitrate (KNO_3)] on anthracnose severity and turf quality (Murphy et al. 2013). Data showed that plots treated with K reduced anthracnose compared to plots treated with N alone, and that combined treatments of K and N further reduced disease severity.

Schmid et al. (2013) conducted a long-term field study to evaluate the effect of soil pH, lime ($CaCO_3$), sulfur (S), and gypsum ($CaSO_4*2H_2O$) on the development of anthracnose on annual bluegrass putting green turf. As soil pH decreased, anthracnose severity increased (Schmid et al. 2013). In particular, soil pH below 5.0 inhibits plant growth, thereby increasing disease incidence (Schmid et al. 2013).

The Golf Course Phytobiome

The United States is home to more golf courses than anywhere else in the world (Breuninger et al. 2013). As of 2012, there were 14,791 eighteen-hole courses in the United States serving approximately 26 million golfers (National Golf Foundation 2012). Aside from serving as a source of recreation, as a whole, golf courses can provide critical wildlife habitat and promote biodiversity. For example, golf courses in North Carolina were found to provide essential habitat for salamanders, both upstream and downstream from managed turf locations (Mackey et al. 2014). Insects and birds also thrive on golf courses (Colding and Folke 2009), with naturalistic areas often supporting many threatened or endangered avian species (Terman 1997). In fact, 829 golf courses from around the world have been designated as Certified Audubon Cooperative Sanctuaries from Audubon International for their role in promoting wildlife and habitat management for birds and other mammals (http://www.audubonintemational.org, accessed 23 September 2015). Likewise, the threatened newt, *Triturus cristatus*, was only found in golf course ponds during a survey of amphibian populations in Sweden (Colding et al. 2009).

Golf courses can also support diverse plant communities. In the United Kingdom, Royal St. George's Golf Course boasts 11 species of wild orchids, including one extremely rare species (Simmons and Jarvie 2001, Gange et al. 2003). In the United States, golf courses have been documented to support diminishing riparian vegetation (Merola-Zwartjes and DeLong 2005), as well as long-leaf pine ecosystems in the southeastern Coastal Plain areas of Virginia and North and South Carolina (Heuberger

and Putz 2003). In Ohio, golf courses have been found to support oak savannahs, areas that are vital for many native woodpecker species (Rodewald et al. 2005).

The ability of golf courses to support ecologically important biota is variable and dependent on a number of factors (Colding et al. 2009). For example, the location of the golf course in respect to naturalized areas, as well as the architectural design, age, and vegetation are just a few of the many characteristics that can positively or negatively impact aboveground flora and fauna (Colding et al. 2009). Regardless, faced with increasing urbanization, many ecologists view golf courses as an opportunity to preserve and encourage rare organisms and support collaboration with golf courses to maintain and promote biodiversity (Terman 1997, Gange et al. 2003, Hodgkison et al. 2006, Colding et al. 2009, Mackey et al. 2014). Success has been achieved by integrating ecologists with golf course developers, as illustrated by a case study in Coachella Valley, California (Holing 1987). Here, wildlife biologists collaborated with golf course developers and state governing agencies to integrate a nature preserve into the resort design, a location that is now home to multiple bird, mammalian, and reptile species, including the endangered fringe-toed lizard (Holing 1987). While such studies demonstrate the vital habitat golf courses can provide to plant and mammals, information regarding the impact that golf courses have on the microbial community that inhabits this ecosystem remains limited.

Microbial Communities in Turfgrass

Microbial communities in grasses are commonly studied, however the focus of such research is often directed at native prairies or grasslands (Johnson et al. 2003, Alster et al. 2013, Fierer et al. 2011, 2012, 2013, Leff et al. 2015), agricultural pastures

currently or previously utilized for livestock (Qi et al. 2011, Leff et al. 2015), or the relationship between endophyte-infected grasses and the surrounding microbial community (Buyer et al. 2011, Casas et al. 2011). Occasionally, sites with cultivated turfgrass are included for comparison to other agronomic systems (Kaye et al. 2005, Han et al. 2007), but they are often not the main foci of such research (Kaye et al. 2005), and as a result, lack important details on how the turf is maintained (Han et al. 2007), making it difficult to draw conclusions about microbial communities in cultivated turfgrass.

Some of the first microbial research specific to the turfgrass ecosystem focused on the effects of specific chemical compounds on microorganisms in turf maintained as lawns. For example, Cole and Turgeon (1977) evaluated the effect of two herbicides (bandane and calcium-arsenate) on the soil (15 cm depth) microbial community in an eight-year-old Kentucky bluegrass stand. They found significantly more bacteria in the soil of bandane treated plots compared to controls, but no difference in plots treated with calcium arsenate (Cole and Turgeon 1977). Likewise, there was no significant difference in fungal populations between treated plots and controls (Cole and Turgeon 1977). Smiley and Craven (1979) found little effect of fungicide use on bacterial and fungal populations in the soil (3 cm depth) of Kentucky bluegrass after three years of applications, except when combination products were used. In this case, combination applications significantly reduced fungal populations and increased bacterial and actinomycete populations (Smiley and Craven 1979).

In addition to pesticides, the effect of fertilizers on microbial communities has also been examined in cultivated turf. In perennial ryegrass, addition of nitrogen or phosphorous had little effect on counts of total bacteria in the rhizoplane, but the

proportion of bacteria capable of degrading chitin did decrease slightly with nutrient addition (Turner et al. 1985). Shortly following, Mancino and Torello (1986) examined populations of denitrifying bacteria in five-year-old Kentucky bluegrass stands maintained in silt or silt-loam soils, and found that addition of nitrate fertilizer did not increase the number of denitrifying bacteria, but silt-based soils maintained higher populations of denitrifying bacteria overall. In six-year-old Kentucky bluegrass amended with various inorganic/organic fertilizers, bacterial and fungal populations were not significantly altered in the soil (6 cm depth), however, bacterial counts did fluctuate significantly among fertilizers in the leaves and thatch (Liu et al. 1995).

The focus of studies examining microbial communities in turfgrass soon shifted to putting greens, as they gained a reputation of being inhospitable for microorganisms due to their lack of organic matter and intensive management practices (Hodges 1990). As a result, there have been several efforts to quantify and identify microorganisms in putting greens using culture-based methods (Mancino et al. 1993, Elliot and Des Jardin 1999a, Elliot and Des Jardin 1999b, Bigelow et al. 2002, Elliot et al. 2003, Elliot et al. 2004). One of the earliest studies, by Mancino et al. (1993), broadly analyzed microbial populations in a creeping bentgrass putting green in Arizona. They revealed more bacteria, fungi, and actinomycetes in the thatch layer than the soil (soil sampled to 7.6 cm depth), and found that these microbial levels were comparable to native soils (Mancino et al. 1993). When they added water-soluble nitrogen, they saw no effect on total counts of bacteria or actinomycetes in the soil or thatch (Mancino et al. 1993). However, fungal counts did increase in the soil (Mancino et al. 1993).

As culturing techniques were refined, more specific groups of microorganisms, at lower taxonomic levels, were identified. For example, populations of fungi, fluorescent pseudomonads, *Stenotrophomonas maltophila*, actinomycetes, and heat-tolerant *Bacillus* species were monitored in a bermudagrass (*Cynodon dactylon* L.) putting green receiving nitrogen from four organic sources and one synthetic source of N (Elliot and Des Jardin 1999). Over two years, only *S. maltophila* counts were significantly different among the natural/synthetic organic fertilizer treatments, and this was detected on only one sampling date (Elliot and Des Jardin 1999). However, this study fails to account for inconsistencies between fertilizer sources in the total amount of P, K and secondary nutrients applied, which may have a greater impact on microbial population than nitrogen form (synthetic vs. natural organic N). Regardless, the study by Elliot and Jardin (1999) was among the first investigations examining specific genera of microbes in putting green turf, setting the stage for several similar studies in this system. For example, Elliot et al. (2003) monitored microbial populations in a creeping bentgrass putting green in Alabama exposed to two rates of urea. Here, counts of *Bacillus sp.,* gram-negative bacteria, and total aerobic bacteria were higher on some sampling dates under high nitrogen treatments (260 kg N ha^{-1} yr^{-1} versus 520 kg N ha^{-1} yr^{-1}) (Elliot et al. 2003). These results reflect year-round applications of urea, as the warmer climate in the southeastern U.S. supports a continuous growing season.

Realizing that many factors can influence microbial populations in the soil, Elliot et al. (2004) employed culturing methods to examine how microbial communities change as a result of turfgrass host or geographic location. Sampling creeping bentgrass putting greens in Alabama and North Carolina, and hybrid bermudagrass putting greens in

Florida and South Carolina, revealed an influence of host plant on bacterial populations, with the roots of creeping bentgrass containing more fluorescent pseudomonads than hybrid bermudagrass (Elliot et al. 2004). Contrastingly, the rhizosphere of bermudagrass putting greens exhibited greater numbers of actinomycetes, Gram-positive bacteria, and heat-tolerant species than bentgrass in this study (Elliot et al. 2004). Geography also appeared to influence bacterial counts, with the North and South Carolina locations always having the highest numbers of bacteria (Elliot et al. 2004). However, the two Carolina sampling sites were from active golf courses, while the Alabama and Florida sites were established at two separate university research centers, thus different management practices could also attribute to the results reported here (Elliot et al. 2004).

Putting green management practices have also been studied for their effects on the rhizosphere microbial community. For example, Bartlett et al. (2008) found that annual bluegrass putting greens contained fewer microbial communities within the top 7.5 cm of the soil than less intensely managed sites, such as fairways and roughs. The authors conclude aggressive management practices on putting greens affect the microbial community (Bartlett et al. 2008), but they fail to account for variations in soil type or geographic locale, as samples were collected from various locations on a golf course in the United Kingdom. Feng et al. (2002) looked at the effect of aeration and trinexapac-ethyl (TE) on rhizosphere microbial communities in Crenshaw and Penncross creeping bentgrass in Alabama, and found that while mycorrhizal infections were higher in TE-treated plants, aeration and TE application did not otherwise impact soil microbial communities.

Putting green age has also been examined for its influence on the resident microbial communities. Newly established creeping bentgrass greens in North Carolina contained initial bacterial counts of 10^6 colony forming units (CFUs), but reached greater than 10^8 CFUs within the first six months (Bigelow et al. 2002). Several populations also appeared to become stable with age, as fluorescent pseudomonad populations were not statistically significant on any of the four sampling dates 16 months after turfgrass establishment (Bigelow et al. 2002). Likewise, actinomycetes and Gram negative bacteria populations were not statistically significant on the last two sampling dates, 23 months after putting green establishment (Bigelow et al. 2002). In Nebraska, analysis of microbial biomass (cellular phospholipids) from 47 putting greens from 12 golf courses revealed a positive relationship between microbial biomass and turfgrass age (Kerek et al. 2002). That is, greens 15 years of age or older displayed more microbial biomass than those younger than 15 years old (Kerek et al. 2002). Likewise, fungi and Gram-negative bacteria increased in the rhizosphere of creeping bentgrass plants established in the greenhouse with time, though plants were only evaluated for 160 days in this study (Steer and Harris 2000). Distinct microbial communities were also apparent in creeping bentgrass putting greens ranging in age from three to thirty years old; however three different golf courses were sampled in this study, thus other factors, such as contrasting soil properties or management practices between the courses could have also contributed to the observed results (Gaulin 2009).

While all of the aforementioned studies provided important insight into the populations of large groups of microorganisms present on golf courses, many relied on culture-based techniques. As a result, only a small subset of the total microbial

community was captured with these methods. For example, studies have estimated that only 0.1% to 1% of soil bacteria can actually be cultured (Torsvik et al. 1990, Amann et al. 1995, Torsvik and Ovreas 2002), thus plating on selective media likely greatly underrepresents total enumerable microbes. Likewise, taxonomic identification was rarely possible beyond classification to broad groups (ex. Actinomycetes, fluorescent pseudomonads) using culture-based methods, thus much of the potential microbial diversity in putting greens remains undescribed.

As techniques to study microbial populations became more advanced, culture-based techniques were supplemented with the analysis of phospholipid fatty acid (PLFA) profiles. PLFA analysis compares the phospholipids that comprise the cellular membranes of known microorganisms to unknown microbes (Frostegard et al. 2010). While PLFA analysis is rapid and relatively inexpensive, it has drawbacks and results can be difficult to interpret (Frostegard et al. 2010). For example, it is difficult to link PLFA profiles to a microorganism's physiological state, as the turnover of PLFA profiles in the soil may not necessarily reflect microbial death (Frosetgard et al. 2010). Furthermore, taxonomic resolution is not always possible with PLFA analysis, as some phospholipid profiles can overlap between microorganisms (Amann et al. 1995, Frosetgard et al. 2010). Elliot et al (2008) found that PLFA analysis of bacterial communities in putting greens in the southeastern U.S. were not able to identify 32 to 50% of genera between sampling sites (2008). More specifically, they found that only five bacterial genera (*Bacillus*, *Clavibacter*, *Flavobacterium*, *Microbacterium*, and *Pseudomonas*) comprised at least 1% of all samples: (Elliot et al. 2008). *Bacillus* dominated bermudagrass greens in Florida and South Carolina, while *Pseudomonas* was dominate in a creeping bentgrass green in

North Carolina (Elliot et al. 2008). In Alabama, *Pseudomonas* and *Bacillus* genera were present in equal proportions in creeping bentgrass putting greens (Elliot et al. 2008).

Nucleic acid-based approaches can provide better taxonomic resolution and more accurately reflect the true microbial community than culturing on selective media or PLFA analysis (Amann et al. 1995). Denaturing gradient gel electrophoresis (DGGE) is a DNA fingerprinting technique that can be used to separate DNA fragments that have the same length, but different nucleotide sequences (Muyzer and Smalla 1998). Using DGGE analysis of the ribosomal RNA (rRNA) region from bacteria and fungi, Sigler and Turco (2002) revealed that chlorothalonil positively impacted five bacterial clones and negatively impacted two bacterial clones in rhizosphere soil obtained from a 1-year-old sand-based putting green. Only two fungal clones were altered as a result of chlorothalonil application, and the response depended on the rate applied. Sequencing of the five positively impacted bacterial clones revealed nine species- *Nevskia ramosa*, *Lysobacter antibioticus*, *Leptothorix discophora*, *Rubrivivax gelatinosus*, *Blastomonas natatora*, *B. ursinicola*, *Agrobacterium rhizogenes*, *Erythrobacter citreus*, and *Pseudomonas cichorii*, while sequencing of the two negatively impacted bacterial clones identified only *Flexibacter flexilis* and *Polyangium cellosum* (Sigler and Turco 2002). For fungi, DNA sequencing identified *Glomus claroideum* and *Chaetomium murorum*, both of which were slightly enhanced at rates of chlorothalonil 0.2 to 1 times the label application rates, although *G. claroideum* was negatively impacted at 5 times the label rate. However, all of the taxonomic identifications in this study are tentative, as some of the rRNA DGGE bands sequenced were relatively short (approximately 194 bp for

bacteria, 200 bp for fungi), and displayed low homology (86%) to sequences in GenBank, making definitive species diagnosis difficult (Sigler and Turco 2002).

A more comprehensive approach was taken by Karp and Nelson (2004), who analyzed a 1 kb region of bacterial 16s rDNA. They sequenced between 150 and 190 bacterial 16s rDNA clones obtained from the rhizosphere of creeping bentgrass established on sand or soil, respectively. Their results show that sand-based root zones were dominated by Gram-negative bacterial species, whereas soil-based root zones exhibited higher overall diversity and contained more Gram-positive species (Karp and Nelson 2004).

These approaches give important insight into the identity of microorganisms present in putting greens, but they provide no information about the current metabolic activities of these microbes, or whether they are alive or dead. Even fewer studies have analyzed microbial function in turfgrass, and these studies have been limited to North Carolina (Dell et al. 2010, Yao et al. 2011). Total microbial respiration has been shown to be higher in warm-season turfgrass stands compared to cool-season turfgrass stands, and N mineralization was reported to be higher in cool-season turf (Yao et al. 2011). However, no information was provided regarding the identity of organisms performing these processes. Dell et al. (2010) examined bacterial *nirK* and *nosZ* genes to gain insight into the denitrification process in bermudagrass fairways and revealed that denitrifying bacterial communities become more diverse and maintain their diversity as turfgrass stands aged, (Dell et al. 2010), but once again definitive species identifications on these organisms was not reported.

Today, there are numerous advanced molecular technologies available to characterize microbial communities in the environment. In particular, next-generation sequencing technologies bypass the need for culturing, allowing a high percentage of the microbial community to be identified from complex environmental samples, such as soil, water and plants, in a relatively fast and inexpensive manner (Shokralla et al. 2012). Analysis of mixed microbial genomes from the environment is known as metagenomics, and makes use of species-specific genomic regions for taxonomic identification of unknown organisms (Schloss and Handlesman 2005, Shokralla et al. 2012). The internal transcribed spacer (ITS) region is commonly used to identify fungi (Schoch et al. 2012), while the 16S ribosomal region is used for archaea (Park et al. 2008) and bacterial species (Sogin et al. 2006). Currently, next-generation metagenomic sequencing studies of environmental systems dominate the literature. In fact, a recent web-based search returned 31,400 citations when using the keyword 'microbial metagenomics' (https://scholar.google.com; accessed 2015 August 24). Yet, next-generation metagenomic sequencing has not been utilized to examine microbial communities in turfgrass, and in particular, golf course putting greens. Employing advanced sequencing technologies in this environment can advance our understanding of microbial communities in golf courses, and provide an important foundation for developing more advanced hypotheses regarding microbial and ecosystem processes in this input-intensive system. This is particularly true for the northeastern U.S., a region that has been underrepresented in previous microbial work in turfgrass.

Summary and Future Directions for Research Studying Microbial Communities in the Turfgrass Environment

Putting greens represent unique, input-intensive environments. They are the most intensely managed sites on golf courses, requiring daily mowing and irrigation, as well as regular fertility inputs and disease management strategies to maintain conditions suitable for the game of golf (Breuninger et al. 2013). Often, many of these inputs are used to control disease or reduce overall severity, but our understanding of the ecology of the microorganisms in putting green turf is limited. For example, anthracnose disease is strongly influenced by fertility, yet very little is known about the distribution of the causal agent, *C. cereale,* in putting green turf, and how fertility may alter the abundance of this important fungus. Similarly, extensive research has focused on controlling anthracnose disease in annual bluegrass putting green turf, but there have been no comprehensive studies examining the resident microbial communities of annual bluegrass putting green turf and how they may interact with *C. cereale*. Likewise, questions regarding management practices and their impact on the community dynamics of the surrounding resident microbial community also remain unanswered. Does the input-intensive nature of putting green turf favor a homogenous microbial population? Is this community dominated by pathogens? And, just as important, can we use these routine management practices to select for a desirable rhizosphere microbial community to promote plant health and productivity? Microbial communities are capable of adapting to soil nutrient content and have been shown to vary in species composition based on changing soil environments (van Diepeningen et al. 2006), thus regular fertility applications may be altering microbial community structure and possibly the distribution

of other pathogenic microorganisms in putting green turf. Assessing microorganisms in putting green turf will give insight into the overall health and productivity of this system, as well as increase our understanding how precision turf management affects the ecology of this unique ecosystem.

In the near future, understanding the turfgrass phytobiome and its constituents will likely become an essential component of turfgrass management. Recently, Quebec and several states (New York, Maryland, California), including New Jersey (New Jersey Act P.L. 2010, c. 112;C.58:10A-64) have passed legislation limiting the timing and rate of fertilizers for applications. While this legislation does not yet apply to golf courses, such laws may be enacted in the future. In cases such as anthracnose, where fertility is relied on for disease management, this could become problematic. Agriculture is faced with a similar demand- meet the needs of a growing population while maintaining sustainability and reducing inputs. As a result, agricultural scientists seek novel ways to reduce disease, maintain healthy soils, and increase yields, all while reducing chemical inputs. This initiative is driving phytobiome research, encompassing entire systems and their microbial constituents, instead of individual plants. This holistic approach has not yet been applied to turfgrass systems, but would be extremely valuable for this system. As turfgrass managers face growing demands to do more with less chemical inputs, it becomes increasingly important to understand the relationship between turfgrass, the resident microbial community it houses, and the practices that may alter it.

The advent of advanced molecular technologies provides a unique opportunity to examine important questions about microorganisms in putting green turf. It is now

possible to characterize entire microbial communities in an environment and begin to understand their function using tools such as next-generation sequencing.

Thesis Overview

In this dissertation, results from a series of investigations examining the microbial communities in annual bluegrass putting green turf will be reported and discussed, beginning with *C. cereale*. As one of the most important fungal pathogens on annual bluegrass in the northeastern U.S., we first sought to determine the distribution of the two lineages (clades) of *C. cereale* in the natural environment. The results of this research are described in Chapter 1. The following chapter describes a field study that was initiated to examine the resident microbial community in annual bluegrass putting green turf exposed to different nitrogen rates over time. Finally, the last chapter examines how nitrogen and potassium affect the resident microbial community in two separate annual bluegrass putting greens naturally infected with *C. cereale*. All three chapters utilize advanced molecular technologies, such as real-time PCR and next-generation sequencing, to evaluate the diversity, distribution, and abundance of microorganisms in annual bluegrass putting green turf. The overall goals of this work were to: 1) increase our understanding of the annual bluegrass putting green microbiome, including common pathogens and benign species, and 2) how this community may be impacted by regular fertility applications. This was done in the hope that knowledge gained from this research would provide insight into how changes in microbial community dynamics may affect annual bluegrass health and possibly tolerance to pests and environmental stress. This research will also serve as a foundation for developing a systems approach for evaluating microbial diversity, plant health, and ecosystem productivity in the turfgrass ecosystem.

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CHAPTER 1: Influence of Host and Geographic Locale on the Distribution of *Colletotrichum cereale* Lineages

ABSTRACT

Colletotrichum cereale is an ascomycete inhabitant of cool-season Pooideae grasses. The fungus has increased in frequency over the past decade as a destructive pathogen of *Poa annua* and *Agrostis stolonifera* turfgrass. *Colletotrichum cereale* exists as two lineages, designated clades A and B, but little is known about the distribution of these clades in natural environments, or what role these subdivisions may play in the trajectory of disease outbreaks. In this study, our objective was to determine the frequency of *C. cereale* clades A and B. To rapidly discriminate between the two *C. cereale* clades, a real-time PCR assay was developed based on the *Apn2* gene. A collection of 700 *C. cereale* pathogens and endophytes from twenty Pooideae grass genera were genotyped. 87% of the collection was identifed as part of clade A, 11.7% as part of clade B, and 1.3% was a mixture. *Colletotrichum cereale* from turfgrass hosts in North America were most commonly members of clade A (78%). The overabundance of clade A in turfgrass isolates was directly attributable to the dominance of this lineage from southern sampling sites, irrespective of host. In contrast, 111 *C. cereale* turfgrass isolates collected from northern sampling sites were evenly distributed between clades A and B. Only 28% of *C. cereale* from *A. stolonifera* at northern sampling sites were part of clade A. These data show that environmental factors such as geographic location and host identity likely played a role in the distribution of the major *C. cereale* clades in North American turfgrass.

INTRODUCTION

Colletotrichum cereale is a widely distributed fungus that lives in association with monocot grasses of the Poaceae subfamily Pooideae (Selby and Manns 1909, Crouch et al. 2009b). The fungus inhabits at least twenty cool-season (C3 photosynthesis) Pooideae genera in numerous ecosystems, including cultivated cereal crops, grasses grown for forage, athletic fields and lawns, and natural landscapes such as prairies and grasslands (Crouch and Beirn 2009, Hyde et al. 2009). Although best known as a pathogen of cultivated grasses, *C. cereale* also survives in host tissue without producing any visible signs of disease (Crouch et al. 2009c). *C. cereale* causes anthracnose disease in parasitized grasses, with symptoms varying based on the host and tissue infected (Crouch and Beirn 2009). Since the initial description of the fungus in 1908, sporadic but notable disease outbreaks caused by *C. cereale* have been documented (Crouch and Beirn 2009). Production of wheat, oats and barley in the United States suffered from severe anthracnose outbreaks during the early part of the 20th century (Crouch and Beirn 2009). More recently, grasses cultivated as turfgrass on golf course putting greens have been plagued by destructive anthracnose disease outbreaks, resulting in substantial economic losses and an undesirable but requisite increase in fungicide usage (Murphy et al. 2008). In turfgrass systems, anthracnose caused by *C. cereale* manifests as either a foliar blight of senescing tissue or a basal stem rot, characterized by blackened, rotted, water-soaked tissue at the base of the plant that eventually leads to host death. Two turfgrass species are primarily affected by anthracnose disease: *Poa annua* and *Agrostis stolonifera* (Crouch and Beirn 2009).

The emergence of *C. cereale* as one of the primary diseases impacting turfgrass health on golf course putting greens has prompted several investigations in recent years pertaining to the identity of the fungus, the structure of populations, and the management factors that influence the development of disease in turfgrass hosts (*e.g.* Crouch et al. 2006, Crouch et al. 2008, Crouch et al. 2009a, Crouch et al. 2009b, Inguagiato et al. 2008, Inguagiato et al. 2009a, Inguagiato et al. 2009b, Inguagiato et al. 2010, Inguagiato et al. 2012, Roberts et al. 2011, Roberts et al. 2012). For most of the 20th century, *C. cereale* was considered conspecific with *C. graminicola*, the fungus responsible for maize anthracnose disease, based on morphological similarities (Wilson 1914). Multi-locus phylogenetic trees established the uniqueness of *C. cereale* (Crouch et al. 2006), and confirmed the utility of hyphal appressoria as a distinguishing character for *C. graminicola* (Sutton 1968). Subsequent work showed that *C. cereale* was the basal taxa in a diverse clade of *Colletotrichum* species associated with grasses of the Poaceae family (Crouch et al. 2009a, 2009b; O'Connell et al. 2012). This assemblage of grass-associated species is collectively referred to as the "graminicola" species aggregate, named after the most prominent member, *C. graminicola* (Cannon et al. 2012). The graminicola aggregate is populated by at least seventeen species, most of which are limited to just one or a few host species, and infect warm-season (C4 physiology) grasses (Crouch et al. 2009b, Crouch et al. 2009c, Crouch and Tomasello-Peterson 2012, Crouch 2014). *C. cereale* stands out within the graminicola aggregate for two reasons: (1) this species is the only known member of the group that infects cool-season grasses; and (2) it is plurivorous, with fourteen genera documented as hosts (Hyde et al. 2009).

The wide-host range of *C. cereale* is misleading, as multi-locus sequence analysis shows that the species is subdivided into eleven populations structured according to host/ecosystem origin (Crouch et al. 2009c). The *C. cereale* populations are distributed across two major lineages, designated clade A and clade B (Crouch et al. 2006, Crouch et al. 2008, Crouch et al. 2009a, Crouch et al. 2009b). *C. cereale* clades A and B exhibit an overlapping host range. Both clades are responsible for anthracnose disease in turfgrass, and have also been associated with Pooid grasses as endophytes (Crouch et al. 2009c). Despite substantial evidence for lineage diversification, significant levels of gene flow link clades A and B, indicating that they are of a single species (Crouch et al. 2006, Crouch et al. 2008a, Crouch et al. 2008b). Clade A is subdivided into ten subpopulations, each corresponding with a single host (*P. annua* or *A. stolonifera*) or ecosystem (turfgrass, cereal crops, or prairie; Crouch et al. 2009c). In contrast, clade B is an exceptionally diverse assemblage of isolates from varied environments and hosts, with no subgroups documented (Crouch et al. 2009c). While the subdivision of clade A into subpopulations appears to be driven by host specialization, the factors shaping the earlier diversification of *C. cereale* into clade A and clade B are unknown. Clade A has traditionally been found in higher numbers and encompassing a larger geographic area than clade B isolates (Crouch et al. 2006; Crouch et al. 2008b, Crouch et al. 2009c). However, this pattern may reflect a bias in sampling rather than structure of natural populations. In this study, our objective was to evaluate a large sample of *C. cereale* isolates to determine the frequency and distribution of clade A and clade B from natural populations, before and after the major turfgrass anthracnose disease outbreaks.

MATERIALS AND METHODS

Colletotrichum cereale and other fungal samples

Supplementary Table S1 summarizes the 700 *Colletotrichum cereale* samples included in the present study. The *C. cereale* samples were derived from four sources: (a) 575 samples were isolates of *C. cereale* established in axenic culture, either new or previously described (Crouch et al. 2006, Crouch et al. 2008, Crouch et al. 2009a, Crouch et al. 2009b); (b) 87 samples were preserved *C. cereale* fungarium specimens consisting of plant tissue colonized by *Colletotrichum* fungi, as diagnosed through the presence of setae; (c) 17 samples were annual bluegrass (*Poa annua*) plants symptomatic for anthracnose disease and showing visible signs of *C. cereale* (setae); and (d) 21 samples were wheat plants (*Triticum aestivum*) asymptomatic for anthracnose disease, but with *C. cereale* setae present on sampled tissue. All wild grown grasses were identified through examination of vegetative features and inflorescences using standard morphological keys for grasses (Hitchcock 1971). Species of cultivated cereals and grasses were identified by color, leaf, and visible inflorescence characteristics. Herbarium materials were identified to host following the original collector identifications and confirmed through morphological examinations using the Hitchcock key.

Thirty-four of the cultured isolates were selected to serve as biological replicates. All biological replicates consisted of separate cultures of a given isolate, from which a separate DNA extraction was performed. Ten samples of non-target fungi grown in pure culture were also included as negative controls, along with eight samples of plant tissue (*Viola* sp. and *Poa pratensis*) and two fungarium specimens where *Colletotrichum* was

present on dicot hosts (*Cucurma longa*, *Malva rotundifolia*) but for which the species *C. cereale* was not expected.

DNA extraction and real-time PCR

Genomic DNA from cultured fungal isolates was extracted using a standard phenol:chloroform protocol as previously described (Crouch et al. 2005). Genomic DNA from fungarium specimens and grass tissue colonized by *C. cereale* was extracted by excising small sections of plant tissue where the fungus was evident (~5-10 cm^2), placing the tissue in a 2ml microcentrifuge tube containing six 2.5 mm glass beads (BioSpec Products, Bartlesville, OK), and shaking in a BioSpec bead-beater (BioSpec Products) on the medium setting for six minutes.

DNA was extracted from the lysed tissue using the Omni Prep DNA Extraction Kit (G-Biosciences, Maryland Heights, MO) according to the manufacturer's protocol; final quantities were assessed using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE).

Target regions for primers and hydrolysis probes for real-time PCR were designed from an alignment of the 750-bp region of the *Apn2* sequencing marker as a template (Crouch et al. 2009c). Primer3 was used to identify optimal sites based on target site DNA properties (http://frodo.wi.mit.edu/primer3/). Two probes, A-Apn2 and B-Apn2, were designed to fall within an 85 or 103 bp PCR amplicon to detect *C. cereale* clade A and clade B isolates, respectively. The two probes differed from one another by 8-bp within the 33-bp probe target site (Fig. 1). The forward primer A-Apn2-F contained one single nucleotide polymorphism (SNP) specific to clade A isolates, and the forward primer B-Apn2-F contained two SNPs specific to clade B isolates. The reverse primer,

Apn2-R, was designed for universal use with both forward primers. Primers and probes were synthesized by Integrated DNA Technologies (Coralville, IA); the sequences are summarized in Table 1. Oligonucleotides synthesized for use as probes were modified 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 (IaBk). An additional internal quencher, ZEN, was positioned in the center of both probes to enhance specificity. DNA stocks from cultured *C. cereale* samples were normalized for use in real-time PCR to 15 ng/µl and DNA extracted from leaf tissue colonized by *C. cereale* were diluted 1:50.

The majority of experiments were performed using the Step One Plus system (Applied Biosystems, Foster City, CA); the Roche Light Cycler 480 (Roche Applied Science, Indianapolis, IN) was used for part of the sample. Experiments were performed in 96 well plates: (a) Step One Plus system: MicroAmp Fast Optical 96-Well Reaction Plate (Applied Biosystems); (b) LightCycler 480 White Multiwell Plate 96 (Roche). Reactions consisted of 20-µl volumes containing the following: 2 µl of sample DNA, 1.25 µl of each primer (20 µM stocks), 2.5 µl probe (2 µM stocks), 10 µl Roche Light Cycler 480 Probes Master Mix, and PCR-grade sterile dH_2O provided with the master mix to volume. The cycling program was as follows: 95 $^{\circ}$C for 120 s, followed by 45 cycles of 95 $^{\circ}$C for 5 s, 60 $^{\circ}$C anneal for 30 s, and 72 $^{\circ}$C extension for 1 s. All reactions were performed a minimum of three times for all samples.

DNA from *C. cereale* isolates from which the *Apn2* marker was sequenced (Crouch et al. 2009c) served as positive controls, and at least one positive control was included with each 96-well plate analyzed. Water blanks were included as negative controls for all plates, and non-target DNA from other fungal species was also tested

(Supplemental Table S2). Positive reactions were scored as those that reached the threshold value prior to cycle 40. Amplification was confirmed for all samples by agarose gel electrophoresis after cycling. Probes were considered specific to *C. cereale* clades if cycle threshold (CT) values were zero for non-target DNA and water controls.

Assay sensitivity was assessed by evaluating two 10-fold dilution curves of genomic DNA extracted from pure cultures of *C. cereale* clade A isolate ANCG 17-15 and *C. cereale* clade B isolate NJ-4990 (Fig 2). Reactions were run on the SmartCycler II (Cepheid, Sunnyvale, CA) using 2.5 µl of each primer (10 µM stocks), 2.5 µl probe (1 µM stock), 5 µl PCR grade water, and Cepheid's Smartmix HM lyophilized PCR master mix. Concentrations ranged from 0.4 pg/µl to 100 ng/µl. Standard curves were generated from the dilutions series data to calculate reaction efficiencies and determine minimum detection levels.

Statistical Analyses

An exact binomial logistic regression model was fit to the clade distribution data using the logistics procedure in Statistical Analysis System software v. 9.3 (SAS Institute, Cary, NC). The model tested for the effects of region, host species, and the interaction of region and host on clade distribution.

RESULTS

Analysis of DNA from cultured Colletotrichum cereale isolates

Dual-labeled hydrolysis probes and primer pairs were designed using an *Apn2* nucleotide sequence alignment as a template to identify fixed nucleotide sites that discriminated between the two major *C. cereale* subgroups responsible for anthracnose disease, clades A and B (Crouch et al. 2006). The lowest level of DNA detection was 4.0 x 10^{-4} pg and 5.0 x 10^{-3} pg, for *C. cereale* clade A and clade B, respectively (Fig. 2). Reaction efficencies were 99.53% (amplification factor = 2) for the clade A assay and 147.74% (amplification factor = 2.48) for the clade B assay.

A sample of 575 cultured isolates of *C. cereale* collected from 20 species of pooid grasses was screened using the *Apn2* real-time PCR assays. 96% of the cultured isolates originated from North America, with 98% of the isolates collected 1998 or later. Data from these reactions are summarized in Table 2 and Supplementary Table S1. Diagnosis for the 34 samples used as biological replicates yielded the same diagnosis for all replicates (Supplementary Table S3). Negative controls, water controls and non-target samples produced C_T values equal to zero.

88% of the cultured *C. cereale* isolates were diagnosed as members of clade A, based on positive calls made using the A-Apn2 assay (avg. C_T = 27.57) and negative calls made using the B-Apn2 assay. Twelve percent of the cultured *C. cereale* isolates were diagnosed as members of clade B, based on positive calls made using the B-Apn2 assay (avg. C_T = 27.59) and negative calls using the A-Apn2 assay. Analysis of *C. cereale* isolate PA-5018-3, previously shown to possess mixed group A/group B RFLP fingerprints (Crouch et al. 2008), produced a positive diagnosis from both the A-Apn2

and B-Apn2 assays (CT=25.92 and 24.99, respectively), consistent with the known molecular type for this isolate. From the 609 cultured *C. cereale* isolates, only one isolate (KS-DGI12) could not be diagnosed using either of the two real-time assays. Visualization of the KS-DGI12 reactions using gel electrophoresis showed that no amplicon was produced in any of the reactions (two probes, six replicates). Assessment of KS-DGI12 DNA using the NanoDrop spectrophotometer showed an overabundance of compounds at A230, which may have interfered with the reactions for this sample.

To test the accuracy of clade assignments made using the A-Apn2 and B-Apn2 real-time PCR assays, diagnoses were compared with genotypes of 85 *C. cereale* isolates for which the nucleotide sequence of the *Apn2* locus was already known (Crouch et al. 2006, Crouch et al. 2008, Crouch et al. 2009a). 100% of diagnoses made using the A-Apn2 and B-Apn2 assays corresponded correctly with the sequenced genotype of these samples.

Analysis of DNA extracted from plant tissue

After establishing baseline sensitivity levels and diagnostic accuracy of the clade A and clade B real-time PCR assays using DNA extracted from pure cultures of *C. cereale*, the assays were tested to determine whether *C. cereale* could be detected directly from heterogenous mixtures of fungus and host DNA extracted from *in planta* samples. Data from these reactions are summarized in Table 2 and Supplementary Table S4.

DNA was extracted from sixteen samples of *Poa annua* maintained in a single 3,344 sq m turfgrass putting green for research purposes (Inguagiato et al. 2008). Sample RWCC was obtained from a golf course in northern New Jersey from a *Poa annua* putting green. Plant tissue samples were symptomatic for anthracnose disease; exhibiting

overall chlorosis and dark, necrotic stems with visible acervuli present. DNA was extracted from plant tissue where visible signs of the fungus were present within 24 hours of harvest from the field. All seventeen symptomatic plant tissue samples tested positive for the presence of *C. cereale*. Eleven of the samples were identified as members of *C. cereale* clade A (avg. C_T = 27.45), and six samples were identified as members of clade B (Avg. C_T = 28.49).

The A-Apn2 and B-Apn2 assays were also used to screen for the *in planta* presence of *C. cereale* from 21 wheat samples where *Colletotrichum* acervuli were visible on the leaf sheaths, in the absence of any visible disease symptoms in the colonized host. The wheat samples had been stored at room temperature (~25^o C) for approximately five years before DNA extractions were performed from *Colletotrichum* colonized plant tissue. All 21 wheat tissue samples were positively diagnosed for *C. cereale* clade A (Average C_T = 26.94) using the A-Apn2 assay; no clade B diagnoses were made from these samples.

Samples of DNA extracted from plants not known to serve as a host for *C. cereale* (*Viola* sp.) and healthy *Poa pratensis* plants were *C. cereale*-negative when tested with the A-Apn2 and B-Apn2 assays.

Analysis of DNA from fungarium specimens

After establishing that the A-Apn2 and B-Apn2 real-time PCR assays could be used to detect *C. cereale* groups from heterogeneous mixtures of DNA derived from host tissue colonized with *Colletotrichum*, DNA was extracted from 87 *C. cereale* fungarium specimens and tested using the assays. Data from these reactions are summarized in Table 2 and Supplementary Table S5. Fungarium specimens consisted of leaf tissue

samples from eighteen Pooid grass species ranging from 70-120 years old. Visible morphological signs of *Colletotrichum* – melanized setae – were observed on all specimens through examination using a stereomicroscope. After DNA extraction from the host/fungal matrix, gel electrophoresis showed DNA was fragmented, 200-bp or less, consistent with standard degradation profiles described for ancient DNA samples (data not shown).

C. cereale was detected from 88.5% of the 87 fungarium specimens. Average C_T values for fungarium samples were 35.43 for clade A (n=57), and 35.47 for clade B (n=6), averaging 7-8 cycles later than from DNA extracts from cultured *C. cereale* isolates. Six fungarium specimens were diagnosed as likely belonging to clade A after visual inspection of the amplification curves, as low fluorescence intensity and late C_T values were observed for these samples when tested against the B-Apn2 assay. Eight fungarium samples produced positive C_T values from both the clade A and clade B assays. Sequence analysis of the resultant amplicon revealed the presence of both the clade A and clade B genotype in these samples (data not shown). The remaining ten fungarium samples identified as *C. cereale* produced no C_T values for either clade A or B, as did the non-target species *Colletotrichum capsici* (BPI 397265) and *Colletotrichum sp.* (BPI 397277). Visualization of the real-time PCR product through agarose gel electrophoresis and the amplification product of PCR reactions performed using primers alone on the negative fungarium samples yielded no detectable amplicons.

Sample-wide frequency of C. cereale subspecific groups

Of the 700 *C. cereale* samples, 98.4% were diagnosed using the A-Apn2 and B-Apn2 real-time PCR assays. All but one of the eleven undiagnosed samples were

fungarium specimens. Of the 689 *C. cereale* with a group diagnosis, 87% of the sample was part of clade A, 11.7% was part of clade B, and 1.3% of the sample produced mixed A/B diagnoses (Table 2).

Table 3 summarizes *C. cereale* membership relative to host origin as diagnosed using the *Apn2* real-time assays. Overall, *C. cereale* clade A samples were observed from all 32 Pooid host species (Table 3). In contrast, *C. cereale* clade B samples were only observed from eight of the Pooid genera.

98% of the *C. cereale* samples in this study originated from within North America. Of the sixteen samples from other geographic locales (Japan, Germany, Australia), all were cultured isolates. Only two *C. cereale* samples from outside North America, CBS 303.69 and CBS 304.69 collected from *Agrostis tenuis* and *Ammophila arenaria*, respectively, in Germany during 1967, were diagnosed by the *Apn2* real-time assay as members of clade B. This diagnosis was consistent with previous nucleotide sequence data for these isolates (Crouch et al. 2009c).

The largest component of the *C. cereale* sample was drawn from cultured isolates of the fungus from the two primary economic hosts in North America – the turfgrasses *Agrostis stolonifera* and *Poa annua* (n=78 and n=191, respectively). Table 4 summarizes the distribution of *C. cereale* from these two hosts in North American samples. The 269 *C. cereale* samples from turfgrass hosts were plant pathogenic isolates, collected from diseased putting greens between 1998 and 2006, after the emergence of the destructive anthracnose disease outbreaks that took place beginning in the mid 1990s (Murphy et al. 2008). When this collection of *C. cereale* turfgrass isolates was typed using the *Apn2* assays, the overall ratio of clade A to clade B isolates was 3.6 to 1 (A=210; B=59). 87%

of all *P. annua* isolates were typed as members of clade A. In contrast, only 56% of the *C. cereale* isolates from *A. stolonifera* hosts were typed as members of clade A.

The dataset of North American turfgrass isolates of *C. cereale* was evaluated according to two broad geographic subdivisions – designated north and south, according to average annual minimum temperature extremes (Table 4). Analysis of clade A and clade B frequencies in these two broadly defined geographic regions showed that the overabundance of clade A isolates across the entire sample from turfgrass hosts was attributable to the *C. cereale* subsample from the southern region. The 158 *C. cereale* isolates collected from southern sites were predominantly members of clade A; only 2.5% of southern isolates were members of *C. cereale* clade B. In contrast, the 111 *C. cereale* isolates sampled from northern sites were almost evenly divided between clade A and group clade B. However, the frequency of the two *C. cereale* subgroups differed between isolates made from *A. stolonifera* and *P. annua*. Northern region isolates of *C. cereale* made from *A. stolonifera* (n=46) were most commonly members of clade B by a factor of 2.4 to 1, whereas isolates made from *P. annua* (n=65) were most commonly members of clade A by a factor of 1.6 to 1.

A clade × region interaction was detected using logistic regression analysis (Table 5). In the southern region of North America, no differences in sampling response were detected among hosts (p=0.79); there was a 96.9% and 97.6% probability of obtaining a clade A isolate when sampling from *A. stolonifera* or *P. annua*, respectively. However, when sampling in the northern region, there was a 28.3% probability of obtaining a clade A isolate from *A. stolonifera*, whereas there was a 66.1% chance of obtaining a clade A isolate when sampling from *P. annua* turf.

DISCUSSION

The primary objective of this study was to examine the frequency of *C. cereale* clades A and B in the natural environment from a large sample of modern and historical specimens from North America. Our results show that clade A is the predominant group in natural populations of *C. cereale*. Furthermore, the frequency with which we observed clade A from historical specimens indicates that clade A has been the dominant *C. cereale* group in North America for at least a century. Despite the abundance of *C. cereale* clade A in the environment, clade B isolates were also identified throughout the entire sample, as part of both modern collections and from cereal crops and grasses dating back to the original 1908 *C. cereale* fungarium specimens. Thus, on the recent time scale, both lineages are endemic to North America, with direct evidence from fungarium specimens documenting their presence in the United States for over a century. This finding is consistent with the high levels of diversity observed for both *C. cereale* clades from previous multi-locus haplotype analysis (Crouch et al. 2009c).

Colletotrichum cereale isolates diagnosed as clade A dominated the overall collection screened in this study, comprising 87% of our samples. When the distribution of clades was evaluated based on host origin, we observed a similar trend for all non-turfgrass *C. cereale* isolates, with clade A outnumbering clade B on all non-turfgrass hosts. Of particular note was the broad host range of *C. cereale* clade A, with isolates from this lineage identified from 32 different Pooid grass species. In contrast, *C. cereale* clade B was only identified from eight Pooid species. On *Aegilops, Agropyron, Ammophila, Anthoxanthum, Axoponus, Festuca, Holcus, Hordeum, Phleum,* and *Polypogon* hosts, the dominance of clade A could be a result of limited sampling on these

hosts, as each host was only represented by one to four samples. Still, the number of clade A isolates on the remaining hosts greatly outnumbers clade B and should not be discounted. Previous haplotype analysis suggests clade A may be the ancestral group for *C. cereale* (Crouch et al. 2006), and the low frequencies and reduced host range from which we observed clade B isolates supports this theory. While additional data is need to confirm the haplotypes present in our sample collection, it is possible that clade B is transitioning to a broader host range.

The overabundance of clade A observed from the overall *C. cereale* collection did not hold true for isolates obtained from turfgrass hosts (*A. stolonifera, P. annua*) when the samples were subdivided based on host origin and broad geographic range. In southern regions of North America, clade A turfgrass isolates accounted for 97.5% of the sample, regardless of the host. In contrast, in northern regions, *C. cereale* clade A and clade B isolates from turfgrass were found in equal numbers. However, closer examination of the northern *C. cereale* isolates showed that the frequency of clades A and B in turfgrass populations may be host dependent. 77% of *C. cereale* isolates from *P. annua* in the northern region were clade A, whereas 60% of the isolates obtained from *A. stolonifera* were clade B. This data, combined with the results from logistic regression analysis, suggests that there is likely a host preference among turfgrass pathogenic isolates of *C. cereale* in northern regions of North America, and warrants future investigation. Several other graminicolous *Colletotrichum* species are known to exhibit host specificity (*e.g. C. graminicola, C. sublineola, C. navitas*). It is possible that we may be observing the transition to host specificity among lineages of *C. cereale* as previously hypothesized (Crouch et al. 2009c).

The distinct frequencies observed from *C. cereale* clade A and clade B turfgrass isolates based on geographic region is an interesting finding, and rigorous fine-scale sampling should be conducted to confirm this theory. Regardless, we cannot ignore that this apparent geographic distribution may also be influenced by temperature and attributed at least in part to the environmental adaptations of the host. *P. annua* does not tolerate heat stress well (Turgeon and Vargas 2003), therefore it may serve as an opportunistic host for *C. cereale* clade A isolates in southern regions where the host is exposed to significant environmental stresses and weakened prior to infection. The optimum temperature for growth of *C. cereale* in culture and on detached leaf assays has been reported to be anywhere between 22°C to 28°C (Wolff 1947, Bruehl and Dickson 1950, Smith 1954, Herting 1982, Hsiang and Khan 2003), whereas optimal infection in the greenhouse has been reported between 15°C-30°C (Wolff 1947), 27°C-33°C (Vargas et al. 1993), and 30°C-33°C (Bolton and Cordukes 1981). To date, a consensus temperature optima for *C. cereale* has not been determined, likely due to difficulties in establishing a repeatable, greenhouse-based inoculation protocol (Murphy et al. 2008) but also possibly because differences in temperature optima for the two clades were not assessed in previous studies. Varying temperature preferences between *C. cereale* lineages could explain the difficulties surrounding the development of an inoculation protocol, and the range of temperatures reported from early studies, and should be investigated. While temperature extremes seem to play an important role in the *C. cereale* pathosystem, we cannot rule out that other factors in the regions, such as soil type and weather events, may be impacting pathogen distribution. Fine-scale, rigorous sampling is needed to further examine these factors.

Our data shows that clade A and B can co-exist together, as mixed infections on individual plants, or on closely situated plants comprising a single stand of grass. Nine samples derived from a single lesion possessed mixed A/B genotypes. Likewise, of the sixteen samples taken from a single *P. annua* research putting green, ten samples were diagnosed as members of clade A and six samples as clade B. On golf course putting greens, *P. annua* is best known for its ability to colonize established *A. stolonifera* stands, resulting in putting greens of mixed host composition. Given the ability of both clades to exist together in a single putting green, combined with our finding that clades A and B seem to exhibit a host preference in northern regions, golf course putting greens comprised of both *A. stolonifera* and *P. annua* may provide a unique host environment for *C. cereale*. This habitat may allow both clades of the fungus to come in frequent, close contact with one another, potentially resulting in new, aggressive strains of the fungus.

The presence of both major *C. cereale* clades in close proximity to one another – either on the same plant, or within a single stand of grass – raises interesting questions about the interactions between these two lineages, the mechanisms involved in sustaining gene flow while at the same time maintaining the distinction between lineages (Crouch et al. 2009c). The sexual cycle for *C. cereale* has never been documented (Crouch and Beirn 2009), yet gene flow is known to have occurred between clades A and B, and between several populations of this species (Crouch et al. 2006, Crouch et al. 2008a, Crouch et al. 2008b, Crouch et al. 2009c). Several other *Colletotrichum* species (*e.g. C. acutatum, C. lindemuthianum*) are known to complete a parasexual cycle that creates diversity and yields new, pathogenic fungal races (Rosada et al. 2010, da Silva Franco et

al. 2011), thus it is possible that *C. cereale* clade A and B isolates may be undergoing a similar process. This would provide a unique evolutionary advantage to pathogenic *C. cereale* isolates, particularly in heavily managed golf course putting greens, where fungicide resistant strains of *C. cereale* are emerging (Wong and Midland 2007, Wong et al. 2007, Wong et al. 2008).

In the current study, we have developed an important assay for the rapid and accurate genotyping of *C. cereale* clades A and B, without the need for time consuming and labor intensive culturing. The biallelic fixation of the targeted A-Apn2 and B-Apn2 probe site for over 100 years demonstrates that utility of *Apn2* as a diagnostic marker for *C. cereale* lineages. This assay provides an accurate, lineage specific identification of *C. cereale* in as little as 45 minutes from DNA extracted directly from infected host tissue. The assay is quantitative, and sensitive enough to detect as little as 4 pg of *C. cereale* DNA from heterogeneous mixtures of host and environmental DNA. This sensitivity and rapid diagnosis is in stark contrast to traditional culturing methods that require surface sterilizations and multiple sub-culturing steps on antibiotic media to eliminate contaminating organisms before yielding a pure culture. From an applied standpoint, this assay could be utilized for clinical diagnoses, to assess pathogen levels from golf course greens, to ensure that seed is pathogen-free, or as an experimental tool to confirm the identity of *C. cereale* clades. For *C. cereale*, we can now use this assay to look at the distribution of clades A and B within a single putting green and to monitor this distribution over time to gain insight about the trajectory of recent anthracnose disease outbreaks in turfgrass.

To our knowledge, this study also marks the first application of real-time PCR experiments for the detection of *Colletotrichum* species from preserved fungarium specimens. The *Apn2* real-time PCR assays were able to reproducibly detect *C. cereale* from small portions of fungarium materials ranging in age from 70 to 120 years old with a high level of success. Previous work with *Colletotrichum* pathogens of warm season grasses – *C. sublineola, C. echinochloae,* and *C. caudatum* – has demonstrated the power of DNA-sequence based approaches to accurately genotype type specimens from fungarium materials >100 years old (Crouch and Tomasello-Peterson 2012, Crouch 2014). Our experiments demonstrate the utility and sensitivity of real-time PCR as a tool to conduct molecular examinations of historical fungal collections for other *Colletotrichum* species. Real-time PCR assays are particularly well suited for fungarium specimens, as they rely on amplification of very short regions of DNA. Since post-mortem degradation and shearing of DNA into fragments <500-bp is ubiquitous in historic specimens (Verkeley et al. 2014), real-time PCR is well suited to the requirements of working with fungarium materials. The development of similar assays for other *Colletotrichum* species where species concepts are currently in a state of flux (Cannon et al. 2012), may prove useful for typification, species delineation, and the examination of temporal changes in these organisms.

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Table 1. Real-time polymerase chain reaction primers and dual labeled hydrolysis probes developed in this study for detection of *Colletotrichum cereale* subspecific groups.

Primer/Probe[a]	Description	Sequence (5' - 3')
A-Apn2-F	Apn2 forward primer, *C. cereale* group A	CCTGCCAAAACACAAGAAAG
B-Apn2-F	Apn2 forward primer, *C. cereale* group B	CTGGGACGTTGTTTTCAGC
Apn2-R	Apn2 reverse primer, *C. cereale* group A and B	GACACCGGAGTATCCTGTCC
A-Apn2P	Probe, *C. cereale* group A	FAM[b]-TTGCGCTGT-ZEN[c]-TTTGGCGGGTAGACGTGATCTAAT-IaBk[d]
B-Apn2P	Probe, *C. cereale* group B	FAM[b]-CTACGCAGT-ZEN[c]-TTTGATGGGTAGGCGTGACCTAAC-IaBk[d]

[a] Primer/probe sets reside within *Apn2* locus
[b] FAM = 6-carboxy-fluorescein fluorescent reporter dye (IDT, Coralville, IA)
[c] ZEN = internal quencher to enhance specificity (IDT, Coralville, IA)
[d] IBFQ = Iowa Black Fluorescent Quencher (IDT, Coralville, IA)

Table 2. Summary of real-time PCR data generated from *Colletotrichum cereale* samples using *Apn2* detection assay for clade A and clade B. 700 samples were evaluated, plus ten additional non-target *Colletotrichum* and other species included as negative controls. Second derivative C_T (cycle threshold) values represent positive samples when the fluorescent signal crosses the amplification threshold prior to cycle 40. Average C_T values are based on a minimum of three technical replicates.

Clade	**Cultured Samples**[a]		**Fungarium Specimens**		***Poa annua* leaf tissue, with visible signs of infection**		***Triticum aestivum* leaf sheaths, with visible signs of infection**		
	Positive Reactions	Avg. C_T[b]	Positive Reactions	Avg. C_T[b]	Positive Reactions	Avg. C_T[b]	Positive Reactions	Avg. C_T[b]	Total Samples
Clade A	503	27.57	57	35.43	11	27.45	21	26.94	592
Clade A likely[c]	1	38.04	6	37.31	0	–	0	–	7
Clade B	69	27.59	6	35.47	6	28.49	0	–	81
Both Clades	1		8	38.19	0	–	0	–	9
Undiagnosed	1	–	10	–	0	–	0	–	11

[a] The 35 biological replicates are not included in the count of positive reactions.
[b] Average C_T values are given as the mean C_T generated from all technical and biological replicates.
[c] Low fluorescence intensity and late C_T values (>40.0) were observed for these samples when tested using the B-Apn2 assay.

Table 3. Summary of the diagnosis of *Colletotrichum cereale* subgroups by host species using the *Apn2* real-time detection assays, A-Apn2 and B-Apn2. Biological replicates and negative controls are not included. Dashes indicate that no samples originating from a given host plant were evaluated.

	Cultured Isolates		**Fungarium Specimens**		***In planta* Samples**	
Host Species	**Clade A**	**Clade B**	**Clade A**	**Clade B**	**Clade A**	**Clade B**
Aegilops cylindrica	1	0	–	–	–	–
Agropyron repens	–	–	1	0	–	–
Agrostis spp. [a]	49	34	4	–	–	–
Ammophila arenaria	1	0	–	–	–	–
Anthoxanthum odoratum	–	–	1	0	–	–
Arrhenatherum elatius	–	–	2	1	–	–
Avena sativa	6	0	9	0	–	–
Axoponus affinis	1	0	–	–	–	–
Bromus spp. [b]	54	0	7	1	–	–
Calamagrostis spp. [c]	29	0	1	–	–	–
Dactylis glomerata	72	8	0	2	–	–
Elymus spp. [d]	86	0	–	–	–	–
Festuca spp. [e]	2	0	–	–	–	–
Holcus lanatus	1	0	1	0	–	–
Hordeum species [f]	–	–	2	0	–	–
Phleum pretense	–	–	2	2	–	–
Poa spp. [g]	165	28	1	1	11	6
Polypogon fugax	1	0	–	–	–	–
Secale cereale	–	–	22	5	–	–
Triticum spp. [h]	39	0	18	2	21	0

[a] *Agrostis* species = *A. alba*, *A. canina*, *A. stolonifera* and *A. tenuis*.
[b] *Bromus* species = *B. inermis*, *B. rigidus* and *B. secalinus*.
[c] *Calamagrostis* species = *C. acutifolia*, *C. epideios* and *C. inexpansa*.
[d] *Elymus* species = *E. canadensis* and *E. virginicus*.
[e] *Festuca* species = *F. elatior* and *F. rubra*.
[f] *Hordeum* species = *H. jubatum* and *H. vulgare*.
[g] *Poa* species = *P. annua* and *P. pratensis*
[h] *Triticum* species = *T. aestivum* and *T. vulgare*

Table 4. Summary of the diagnosis of *Colletotrichum cereale* subgroups by geographic locale using the *Apn2* real-time detection assays, A-Apn2 and B-Apn2. Diagnoses are given for isolates of *C. cereale* made from diseased turfgrass hosts *Agrostis stolonifera* and *Poa annua*. Diagnoses are separated according to the geographic locale where the sample originated, broken down by states in the United States of America and provinces in Canada. States/provinces are subdivided into regions according to minimum extreme temperature of location following the USDA-ARS Plant Hardiness Zones. Southern region sampling sites were defined as those sites that fell within USDA-ARS Plant Hardiness Zones 7a to 9a (minimum extreme temperature range -17.8 to -15 ^{o}C and -6.7 to -3.9^{o}C, respectively). Northern region sample sites were defined as those sites that fell within USDA-ARS Plant Hardiness Zones 6a or colder (minimum extreme temperature range -23.3 to -20.5 ^{o}C or less). Biological replicates and negative controls are not included. Dashes indicate that no samples originating from a given host plant were evaluated.

	Agrostis stolonifera		***Poa annua***		**Total**	
State/Province	**Clade A**	**Clade B**	**Clade A**	**Clade B**	**Clade A**	**Clade B**
Southern region						
Alabama, USA	4	0	–	–	4	0
California, USA	–	–	96	1	96	1
Mississippi, USA	6	0	–	–	6	0
North Carolina, USA	12	1	27	2	39	3
Tennessee, USA	4	0	–	–	4	0
Texas, USA	1	0	–	–	1	0
Virginia, USA	4	0	1	–	5	0
Total: Southern region	31	1	123	3	154	4
Northern region						
British Columbia, CA	–	–	1	0	1	0
Connecticut, USA	2	4	5	1	7	5
Massachusetts, USA	2	3	1	4	3	7
New Brunswick, CA	–	–	1	0	1	0
New Hampshire, USA	–	–	1	0	1	0
New Jersey, USA	–	–	15	3	15	3
New York, USA	1	1	4	1	5	2
Ontario, CA	7	24	2	2	9	26
Pennsylvania, USA	0	1	11	9	11	10
Rhode Island, USA	1	0	2	2	3	2
Total: Northern region	13	33	43	22	56	55
Total, all North America	44	34	166	25	210	59

Table 5. Exact logistic regression results on the probability of samples diagnosed as *Colletotrichum cereale* clade A across regions and turfgrass hosts.

	Logit	**Odds**	**Probability**	***P* Value**
Southern region				
Agrostis stolonifera	3.4337	30.9911	0.9687	0.7916
Poa annua	3.7136	41.0012	0.9762	0.7916
Northern region				
Agrostis stolonifera	-0.9316	0.3939	0.2826	<0.0001
Poa annua	0.6702	1.9546	0.6615	<0.0001

Supplemental Table S1. Cultured Samples of *Colletotrichum cereale* tested to determine clade membership (A or B) using real-time PCR assays. CT=cycle threshold.

Isolate	Average CT Clade A assay	Average CT Clade B assay	Diagnosis	*Host Species*	Year Isolated	Collector	Origin	Location
00119	34.35	-	A	*Agrostis stolonifera*	2000	T. Hsiang	Canada	Downsview, ON
00137	30.38	-	A	*Agrostis stolonifera*	2000	T. Hsiang	Canada	Osprey Valley, ON
00164	26.75	-	A	*Agrostis stolonifera*	2000	T. Hsiang	Canada	Osprey Valley, ON
00168	32.13	-	A	*Agrostis stolonifera*	2000	T. Hsiang	Canada	Osprey Valley, ON
00176	21.81	-	A	*Agrostis stolonifera*	2000	T. Hsiang	Canada	Osprey Valley, ON
99362	27.93	-	A	*Agrostis stolonifera*	1999	T. Hsiang	Canada	Guelph, ON
99365	29.40	-	A	*Agrostis stolonifera*	1999	T. Hsiang	Canada	Guelph, ON
99370	27.94	-	A	*Poa annua*	1999	T. Hsiang	Canada	Erin, ON
99375	28.51	-	A	*Poa annua*	1999	T. Hsiang	Canada	Erin, ON
99409	37.20	-	A	*Poa annua*	1999	T. Hsiang	Canada	British Columbia
007 BI-2	27.02	-	A	*Agrostis stolonifera*	2006	M. Tomaso-Peterson	Alabama	Birmingham
007 I-2	29.17	-	A	*Agrostis stolonifera*	2006	M. Tomaso-Peterson	Alabama	Birmingham
007 T4-2	25.68	-	A	*Agrostis stolonifera*	2006	M. Tomaso-Peterson	Alabama	Birmingham
007 Z5-2	27.44	-	A	*Agrostis stolonifera*	2006	M. Tomaso-Peterson	Alabama	Birmingham
1039-FS	27.56	-	A	*Festuca sp.*	1984	D. TeBeest	Arkansas	Baldwin Springs
1050-AC	25.05	-	A	*Aegilops cylindrica*	1985	D. TeBeest	Arkansas	Washington Co.
1050AC (duplicate)	26.35	-	A	*Aegilops cylindrica*	1985	D. TeBeest	Arkansas	Washington Co.
279CGCT7	24.19	-	A	*Agrostis stolonifera*	2006	J.E. Kaminski	Connecticut	
289CGMA5	23.79	-	A	*Agrostis stolonifera*	2006	J.E. Kaminski	Massachusetts	
297CGCT7	21.07	-	A	*Agrostis stolonifera*	2006	J.E. Kaminski	Connecticut	
305429PF	27.19	-	A	*Polypogon fugax*	1977	MAFF 305429	Japan	Saga Prefecture
AHCC 10-10	29.56	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 10-13	26.84	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 10-14	32.43	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia

Isolate	Average CT Clade A assay	Average CT Clade B assay	Diagnosis	*Host Species*	Year Isolated	Collector	Origin	Location
AHCC 10-17	29.01	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 10-18	33.88	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 10-2	34.13	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 10-22	27.06	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 10-25	35.60	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 10-35	30.57	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 10-4	24.71	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 10-40	29.38	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 10-42	28.72	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 10-48	31.50	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 10-49	27.83	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 10-5	35.73	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 10-51	25.94	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 10-53	28.18	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 10-54	31.09	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 10-56	27.50	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 10-57	30.03	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 10-6	31.76	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 10-60	27.10	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 10-60 (duplicate)	28.71	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 10-61	33.38	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 10-62	30.20	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 10-63	29.60	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 10-64	26.56	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 10-65	26.50	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 10-69	28.25	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia

Isolate	Average CT Clade A assay	Average CT Clade B assay	Diagnosis	*Host Species*	Year Isolated	Collector	Origin	Location
AHCC 10-71	28.88	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 10-72	26.23	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 10-73	36.74	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 10-74	31.34	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 10-76	34.18	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 10-78	29.73	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 10-83	29.42	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 80	28.43	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 80 (duplicate)	25.55	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 81	28.37	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 81 (duplicate)	26.57	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 82	24.36	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 83	25.46	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 84	25.46	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
ANCG 17-11	34.34	-	A	*Poa annua*	2004	F.P. Wong	California	Pasadena
ANCG 17-13	32.81	-	A	*Poa annua*	2004	F.P. Wong	California	Pasadena
ANCG 17-13	25.30	-	A	*Poa annua*	2004	F.P. Wong	California	Pasadena
ANCG 17-14	33.73	-	A	*Poa annua*	2004	F.P. Wong	California	Pasadena
ANCG17-15	23.57	-	A	*Poa annua*	2004	F.P. Wong	California	Pasadena
ANCG17-15 (duplicate)	24.11	-	A	*Poa annua*	2004	F.P. Wong	California	Pasadena
BRIDGES 1-3	29.27	-	A	*Poa annua*		F.P. Wong	California	
CBS 148.34	23.49	-	A	*Avena sativa*	1934	CBS 148.34	Canada	Alberta
CBS 148.34 (duplicate)	24.83	-	A	*Avena sativa*	1934	CBS 148.34	Canada	Alberta
CBS 240.49	31.66	-	A	*Avena sativa*	1949	CBS 240.49	Germany	
CBS 240.49 (duplicate)	22.45	-	A	*Avena sativa*	1949	CBS 240.49	Germany	

Isolate	Average CT Clade A assay	Average CT Clade B assay	Diagnosis	*Host Species*	Year Isolated	Collector	Origin	Location
CL9	25.63	-	A	*Poa annua*		F.P. Wong	California	
CT-14	25.77	-	A	*Poa annua*	1998	N. Jackson	Connecticut	
CT-19	38.86	-	A	*Agrostis stolonifera*	1998	N. Jackson	Connecticut	Greenwich
CT-2	22.03	-	A	*Poa annua*	1998	N. Jackson	Connecticut	
CT-2 (duplicate)	28.41	-	A	*Poa annua*	1998	N. Jackson	Connecticut	
CT-6956	25.90	-	A	*Poa annua*	2005	J.A. Crouch	Connecticut	
D11426-5	27.54	-	A	*Poa annua*	2005	J.A. Crouch	New Jersey	Wayne
D11727-C1	30.39	-	A	*Poa annua*	2006	J.A. Crouch	New Jersey	Wayne
D11727-C5	30.04	-	A	*Poa annua*	2006	J.A. Crouch	New Jersey	Wayne
D11727-D2	32.46	-	A	*Poa annua*	2006	J.A. Crouch	New Jersey	Wayne
D11809-3	32.20	-	A	*Poa annua*	2006	J.A. Crouch	New Jersey	Belle Meade
D8237	26.53	-	A	*Poa annua*	2005	J.A. Crouch	Pennsylvania	Ambler
D8467	36.27	-	A	*Poa annua*	2005	J.A. Crouch	New Jersey	Milford
D8595	28.45	-	A	*Poa annua*	2005	J.A. Crouch	Pennsylvania	Havertown
D8627	30.03	-	A	*Poa annua*	2005	J.A. Crouch	New Jersey	Manalapan
D8628	25.95	-	A	*Poa annua*	2005	J.A. Crouch	New Jersey	Manalapan
D8810	28.21	-	A	*Poa annua*	2005	J.A. Crouch	Connecticut	Darien
D8853	32.67	-	A	*Poa annua*	2005	J.A. Crouch	New Jersey	Hopewell
D8900	27.11	-	A	*Poa annua*	2005	J.A. Crouch	New York	Hastings-on-Hudson
D8977	26.90	-	A	*Poa annua*	2005	J.A. Crouch	Virginia	Reston
D9559	26.55	-	A	*Poa annua*	2005	J.A. Crouch	New York	Bronxville
EG15	20.09	-	A	*Poa annua*	2003	F.P. Wong	California	Corona
EG25	29.02	-	A	*Poa annua*	2003	F.P. Wong	California	Corona
EG5	28.61	-	A	*Poa annua*	2003	F.P. Wong	California	Corona
FUGC 1	28.31	-	A	*Poa annua*	2003	F.P. Wong	California	Fullerton
FUGC 11-44	27.95	-	A	*Poa annua*	2003	F.P. Wong	California	Fullerton

Isolate	Average CT Clade A assay	Average CT Clade B assay	Diagnosis	*Host Species*	Year Isolated	Collector	Origin	Location
FUGC 11-45	27.49	-	A	*Poa annua*	2003	F.P. Wong	California	Fullerton
FUGC11-43	23.73	-	A	*Poa annua*	2003	F.P. Wong	California	Fullerton
GBGC1	30.84	-	A	*Agrostis stolonifera*	2005	L.P. Tredway	Tennessee	Gatlinburg
GBGC4	25.71	-	A	*Agrostis stolonifera*	2005	L.P. Tredway	Tennessee	Gatlinburg
GBGC5	22.70	-	A	*Agrostis stolonifera*	2005	L.P. Tredway	Tennessee	Gatlinburg
IL-BI-3.5	37.01	-	A	*Bromus inermis*	2005	J.A. Crouch	Illinois	Chicago
IL-BI3.5	24.80	-	A	*Bromus inermis*	2005	J.A. Crouch	Illinois	Chicago
IL-BIKS-20B-I	35.14	-	A	*Bromus inermis*	2005	J.A. Crouch	Illinois	Chicago
IL-C1-7.3A1	26.52	-	A	*Calamagrostis inexpansa*	2005	J.A. Crouch	Illinois	Markham
IL-C1-7.3AS	29.13	-	A	*Calamagrostis inexpansa*	2005	J.A. Crouch	Illinois	Markham
IL-CI-7-3D	25.03	-	A	*Calamagrostis inexpansa*	2005	J.A. Crouch	Illinois	Markham
IMI279189	25.80	-	A	*Axoponus affinis*	1983	IMI279189	Australia	Queensland
KS-10-EC16.3	24.06	-	A	*Elymus canadensis*	2005	J.A. Crouch	Kansas	Manhattan
KS-10-EC1B3	22.97	-	A	*Elymus canadensis*	2005	J.A. Crouch	Kansas	Manhattan
KS-10-EC1C1	23.58	-	A	*Elymus canadensis*	2005	J.A. Crouch	Kansas	Manhattan
KS-10-EC1D2	23.88	-	A	*Elymus canadensis*	2005	J.A. Crouch	Kansas	Manhattan
KS-10-EC1D3	23.92	-	A	*Elymus canadensis*	2005	J.A. Crouch	Kansas	Manhattan
KS-10-EC1E1	23.60	-	A	*Elymus canadensis*	2005	J.A. Crouch	Kansas	Manhattan
KS-10-EC1E3	24.48	-	A	*Elymus canadensis*	2005	J.A. Crouch	Kansas	Manhattan
KS-10-EC1F1	24.02	-	A	*Elymus canadensis*	2005	J.A. Crouch	Kansas	Manhattan
KS-10-EC1F3	26.53	-	A	*Elymus canadensis*	2005	J.A. Crouch	Kansas	Manhattan
KS-10-EC1G1	24.46	-	A	*Elymus canadensis*	2005	J.A. Crouch	Kansas	Manhattan
KS-10-EC1G2	24.93	-	A	*Elymus canadensis*	2005	J.A. Crouch	Kansas	Manhattan
KS-10-EC1H1	24.27	-	A	*Elymus canadensis*	2005	J.A. Crouch	Kansas	Manhattan
KS-10-EC2A1	24.29	-	A	*Elymus canadensis*	2005	J.A. Crouch	Kansas	Manhattan

Isolate	Average CT Clade A assay	Average CT Clade B assay	Diagnosis	*Host Species*	Year Isolated	Collector	Origin	Location
KS-10-EC2A2	26.23	-	A	*Elymus canadensis*	2005	J.A. Crouch	Kansas	Manhattan
KS-10-EC2A3	25.69	-	A	*Elymus canadensis*	2005	J.A. Crouch	Kansas	Manhattan
KS-10-EC2C1	25.12	-	A	*Elymus canadensis*	2005	J.A. Crouch	Kansas	Manhattan
KS-10-EC2C2 (duplicate)	25.21	-	A	*Elymus canadensis*	2005	J.A. Crouch	Kansas	Manhattan
KS-10-EC2D2	24.54	-	A	*Elymus canadensis*	2005	J.A. Crouch	Kansas	Manhattan
KS-10-EC2H2	24.84	-	A	*Elymus canadensis*	2005	J.A. Crouch	Kansas	Manhattan
KS-10-EC2H3	25.80	-	A	*Elymus canadensis*	2005	J.A. Crouch	Kansas	Manhattan
KS-10-EC3	26.48	-	A	*Elymus canadensis*	2005	J.A. Crouch	Kansas	Manhattan
KS-10-EC362	27.32	-	A	*Elymus canadensis*	2005	J.A. Crouch	Kansas	Manhattan
KS-10-EC3A1	25.51	-	A	*Elymus canadensis*	2005	J.A. Crouch	Kansas	Manhattan
KS-10-EC3B2	23.83	-	A	*Elymus canadensis*	2005	J.A. Crouch	Kansas	Manhattan
KS-10-EC3B3	24.38	-	A	*Elymus canadensis*	2005	J.A. Crouch	Kansas	Manhattan
KS-10-EC3D1	24.98	-	A	*Elymus canadensis*	2005	J.A. Crouch	Kansas	Manhattan
KS-10-EC3D2	22.62	-	A	*Elymus canadensis*	2005	J.A. Crouch	Kansas	Manhattan
KS-10-EC3D3	22.25	-	A	*Elymus canadensis*	2005	J.A. Crouch	Kansas	Manhattan
KS-10-EC3E1	25.48	-	A	*Elymus canadensis*	2005	J.A. Crouch	Kansas	Manhattan
KS-10-EC3E2	23.73	-	A	*Elymus canadensis*	2005	J.A. Crouch	Kansas	Manhattan
KS-10-EC3E2 (duplicate)	24.13	-	A	*Elymus canadensis*	2005	J.A. Crouch	Kansas	Manhattan
KS-10-EC3E3	29.32	-	A	*Elymus canadensis*	2005	J.A. Crouch	Kansas	Manhattan
KS-10-EC3F1	25.40	-	A	*Elymus canadensis*	2005	J.A. Crouch	Kansas	Manhattan
KS-10-EC3F2	25.18	-	A	*Elymus canadensis*	2005	J.A. Crouch	Kansas	Manhattan
KS-10-EC3F3	24.99	-	A	*Elymus canadensis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DG01	27.47	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DG04	26.31	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DG05	28.05	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan

Isolate	Average CT Clade A assay	Average CT Clade B assay	Diagnosis	*Host Species*	Year Isolated	Collector	Origin	Location
KS-20-DG14	26.81	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGA1	24.06	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGA2	24.86	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGA3	23.30	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGA4	26.15	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGA5	25.42	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGD1	24.82	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGD2	27.92	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGD3	27.04	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGD4	24.21	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGD5	26.29	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGD5 (duplicate)	25.34	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGF1	24.26	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGF2	24.27	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGF3	25.14	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGF4	24.40	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGF5	25.12	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGG2	27.90	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGG3	32.25	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGG4	29.45	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGG5	28.38	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGH2	24.60	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGH3	28.02	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGH4	24.82	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGH5	24.58	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGI4	26.18	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan

Isolate	Average CT Clade A assay	Average CT Clade B assay	Diagnosis	*Host Species*	Year Isolated	Collector	Origin	Location
KS-20-DGK2	27.36	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGK3	26.64	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGK4	25.25	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGK5	28.75	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGP1	25.02	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGP3	25.32	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGP4	23.82	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGP5	24.62	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGQ1	31.46	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGQ2	29.42	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGQ3	27.50	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGQ4	25.63	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGQ5	26.24	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGR4	24.47	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGR5	25.35	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGS3	28.07	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGS4	28.87	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGS5	29.54	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGSI	32.11	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGV1	25.27	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGV2	25.82	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGV2 (duplicate)	25.66	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGV3	24.83	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGV4	26.49	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGW2	22.95	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGW3	24.71	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan

Isolate	Average CT Clade A assay	Average CT Clade B assay	Diagnosis	*Host Species*	Year Isolated	Collector	Origin	Location
KS-20-DGW4	24.58	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGW5	24.72	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGX1	24.90	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGX2	28.28	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGX3	25.74	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGX5	25.32	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGXV	22.62	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGZ2	34.25	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGZ2	29.91	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGZ3	25.45	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGZ4	31.11	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGZ5	25.46	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGZI	24.52	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EV12	22.96	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EV13	23.07	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EV14	24.35	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EV1D1	31.53	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EV5	39.22	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVA1	27.25	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVA2	25.19	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVA3	24.63	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVC1	27.80	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVC2	26.95	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVC3	23.24	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVD1	24.36	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVD2	26.28	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan

Isolate	Average CT Clade A assay	Average CT Clade B assay	Diagnosis	*Host Species*	Year Isolated	Collector	Origin	Location
KS-20-EVD2 (duplicate)	35.46	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVD3	29.16	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVD4	23.66	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVD5	28.15	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVEF	33.70	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVI1	23.81	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVI5	33.77	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVJ1	25.61	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVJ2	25.33	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVJ3	25.18	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVK5	24.31	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVL3	25.91	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVM	32.95	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVM1	26.47	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVM2	26.55	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVM3	26.17	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVN1	26.68	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVN3	25.91	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVQ1	23.41	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVQ2	23.70	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVQ3	24.81	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVQ5	35.61	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVR1	24.26	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVR2	33.64	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVT1	29.16	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVT3	26.79	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan

Isolate	Average CT Clade A assay	Average CT Clade B assay	Diagnosis	*Host Species*	Year Isolated	Collector	Origin	Location
KS-20-EVT4	22.53	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVT5	24.15	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVU1	26.54	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVV1	25.54	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVV1 (duplicate)	25.82	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVV2	24.52	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVV4	35.19	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVV5	24.59	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVW2	33.95	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVW3	26.55	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVX1	27.72	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVX2	32.95	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVX3	27.67	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVX4	30.97	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVY2	25.24	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVZ4	26.27	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-BIG	28.21	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-DGU	26.46	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-DGY	26.17	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-I11	26.22	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-I13	30.03	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-I15	26.68	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-IA2	26.55	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-IA3	26.49	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-IA4	27.68	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-IA5	36.22	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan

Isolate	Average CT Clade A assay	Average CT Clade B assay	Diagnosis	*Host Species*	Year Isolated	Collector	Origin	Location
KS-20B-IB3	29.03	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-ID1	24.78	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-ID2	25.14	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-IE1	25.54	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-IE2	37.21	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-IE3	24.91	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-IF2	28.31	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-IF3	25.08	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-IF4	24.62	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-IF5	25.24	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-IG1	27.03	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-IG2	25.77	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-IG3	26.06	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-IH2	26.59	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-II1	21.86	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-II2	27.50	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-II3	26.87	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-II4	29.36	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-II5	27.73	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-IKS	25.78	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-IM1	25.68	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-IM2	25.94	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-IM3	24.43	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-IN1	24.92	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-IN2	25.83	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-IN3	24.77	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan

Isolate	Average CT Clade A assay	Average CT Clade B assay	Diagnosis	*Host Species*	Year Isolated	Collector	Origin	Location
KS-20B-IO3	26.61	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-IP1	26.94	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-IP2	27.06	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-IP3	26.89	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-IP4	27.55	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-IP5	25.96	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-IQ1	26.79	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-IR2	24.61	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-IR3	24.67	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-IS1	27.14	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-IS2	27.67	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-IS3	26.69	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-IS4	25.10	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-20B-IS5	25.49	-	A	*Bromus inermis*	2005	J.A. Crouch	Kansas	Manhattan
KS-4-4-W4	26.69	-	A	*Triticum aestivum*	2005	J.A. Crouch	Kansas	Manhattan
KS-FE7A4	25.69	-	A	*Festuca elatior*	2005	J.A. Crouch	Kansas	Manahattan
KS-FE7A4 (duplicate)	22.70	-	A	*Festuca elatior*	2005	J.A. Crouch	Kansas	Manahattan
KS-TA 1.4C	30.84	-	A	*Triticum aestivum*	2005	J.A. Crouch	Kansas	Manahattan
KS-TA 1.4D	26.02	-	A	*Triticum aestivum*	2005	J.A. Crouch	Kansas	Manahattan
KS-TA 10.1	30.61	-	A	*Triticum aestivum*	2005	J.A. Crouch	Kansas	Shawnee Co.
KS-TA 10.1A	27.70	-	A	*Triticum aestivum*	2005	J.A. Crouch	Kansas	Shawnee Co.
KS-TA 10.8	30.25	-	A	*Triticum aestivum*	2005	J.A. Crouch	Kansas	Shawnee Co.
KS-TA 36.2B	25.08	-	A	*Triticum aestivum*	2005	J.A. Crouch	Kansas	Shawnee Co.
KS-TA 5.5-1	28.37	-	A	*Triticum aestivum*	2005	J.A. Crouch	Kansas	
KS-TA-F1 W4E15	37.86	-	A	*Triticum aestivum*	2005	J.A. Crouch	Kansas	
KS-TA-F14 E3	28.40	-	A	*Triticum aestivum*	2005	J.A. Crouch	Kansas	

Isolate	Average CT Clade A assay	Average CT Clade B assay	Diagnosis	*Host Species*	Year Isolated	Collector	Origin	Location
KS-TA-F14 W4F	28.22	-	A	*Triticum aestivum*	2005	J.A. Crouch	Kansas	
KS-TA-F14 W6A1.3-1	24.11	-	A	*Triticum aestivum*	2005	J.A. Crouch	Kansas	
KS-TA-F14 W6A1.3-2	29.32	-	A	*Triticum aestivum*	2005	J.A. Crouch	Kansas	
KS-TA-F14 W6A1.3-3	23.87	-	A	*Triticum aestivum*	2005	J.A. Crouch	Kansas	
KS-TA-F14 W6A1.3-4	30.03	-	A	*Triticum aestivum*	2005	J.A. Crouch	Kansas	
KS-TA-F14 WE1-5	28.01	-	A	*Triticum aestivum*	2005	J.A. Crouch	Kansas	
KS-TA-F15 W16.3.5	30.68	-	A	*Triticum aestivum*	2005	J.A. Crouch	Kansas	Jewell Co.
KS-TA-F15 W16A	33.14	-	A	*Triticum aestivum*	2005	J.A. Crouch	Kansas	Jewell Co.
KS-TA-F3 2.1.3	36.11	-	A	*Triticum aestivum*	2005	J.A. Crouch	Kansas	
KS-TA-F9 W1.2-4	31.58	-	A	*Triticum aestivum*	2005	J.A. Crouch	Kansas	
KS-TA-F9 W4-2.4 (duplicate)	30.33	-	A	*Triticum aestivum*	2005	J.A. Crouch	Kansas	
KS-TA15116A	21.68	-	A	*Triticum aestivum*	2005	J.A. Crouch	Kansas	Manhattan
MA-11	21.09	-	A	*Poa annua*	1998	N. Jackson	Massachusetts	
MA-11 (duplicate)	21.07	-	A	*Poa annua*	1998	N. Jackson	Massachusetts	
MA-21	31.61	-	A	*Agrostis stolonifera*	1998	N. Jackson	Massachusetts	
MAFF236902	23.59	-	A	*Agrostis stolonifera*	1993	MAFF236902	Japan	
MAFF236961	23.92	-	A	*Agrostis stolonifera*	1993	MAFF236961	Japan	
MAFF305076	22.73	-	A	*Avena sativa*	1966	MAFF305076	Japan	Saga Prefecture
MAFF305371	24.77	-	A	*Avena sativa*	1966	MAFF305371	Japan	Saga Prefecture
MAFF305384	22.20	-	A	*Holcus lanatus*	1972	MAFF305384	Japan	Fukushima Prefecture
MAFF305427	27.10	-	A	*Avena sativa*	1977	MAFF305427	Japan	Kumamota Prefecture
MAFF305427 (duplicate)	23.62	-	A	*Avena sativa*	1977	MAFF305427	Japan	Kumamota Prefecture
MAFF305429	23.56	-	A	*Polypogon fugax*	1977	MAFF305429	Japan	Saga Prefecture
MAFF305432	22.06	-	A	*Dactylis glomerata*	1977	MAFF305432	Japan	Saga Prefecture

Isolate	Average CT Clade A assay	Average CT Clade B assay	Diagnosis	*Host Species*	Year Isolated	Collector	Origin	Location
MAFF305436	23.73	-	A	*Dactylis glomerata*	1977	MAFF305436	Japan	Tochigi Prefecture
MAFF305436 (duplicate)	20.72	-	A	*Dactylis glomerata*	1977	MAFF305436	Japan	Tochigi Prefecture
MAFF305436 (duplicate)	25.23	-	A	*Dactylis glomerata*	1977	MAFF305436	Japan	Tochigi Prefecture
MAFF511114	23.92	-	A	*Avena sativa*	1977	MAFF511114	Japan	Kumamoto Prefecture
MAFF511140	22.46	-	A	*Dactylis glomerata*	1977	MAFF511140	Japan	Tochigi Prefecture
NBR-13	24.32	-	A	*Poa annua*	1998	N. Jackson	Canada	New Brunswick
NBR-13 (duplicate)	25.66	-	A	*Poa annua*	1998	N. Jackson	Canada	New Brunswick
NC ABR15	39.97	-	A	*Agrostis stolonifera*	2005	L.P. Tredway	North Carolina	Sanford
NC ABR16	25.06	-	A	*Agrostis stolonifera*	2005	L.P. Tredway	North Carolina	Sanford
NC ABR17	25.75	-	A	*Poa annua*	2005	L.P. Tredway	North Carolina	Blowing Rock
NC ABR2	26.01	-	A	*Agrostis stolonifera*	2005	L.P. Tredway	North Carolina	Wilmington
NC ABR21	40.09	-	A	*Poa annua*	2005	L.P. Tredway	North Carolina	Blowing Rock
NC ABR23	23.43	-	A	*Poa annua*	2005	L.P. Tredway	North Carolina	Blowing Rock
NC ABR26	26.65	-	A	*Poa annua*	2005	L.P. Tredway	North Carolina	Blowing Rock
NC ABR31	29.18	-	A	*Poa annua*	2005	L.P. Tredway	North Carolina	Blowing Rock
NC ABR32	34.15	-	A	*Poa annua*	2005	L.P. Tredway	North Carolina	Blowing Rock
NC ABR4	27.68	-	A	*Agrostis stolonifera*	2005	L.P. Tredway	North Carolina	Wilmington
NC ABR41	25.54	-	A	*Poa annua*	2005	L.P. Tredway	North Carolina	Blowing Rock
NC ABR42	26.01	-	A	*Poa annua*	2005	L.P. Tredway	North Carolina	Blowing Rock
NC ABR43	27.40	-	A	*Poa annua*	2005	L.P. Tredway	North Carolina	Blowing Rock
NC ABR44	28.92	-	A	*Poa annua*	2005	L.P. Tredway	North Carolina	Blowing Rock
NC ABR46	26.12	-	A	*Agrostis stolonifera*	2005	L.P. Tredway	North Carolina	Monroe
NC ABR47	27.17	-	A	*Agrostis stolonifera*	2005	L.P. Tredway	North Carolina	Monroe
NC ABR49	26.08	-	A	*Agrostis stolonifera*	2005	L.P. Tredway	North Carolina	Monroe
NC ABR5	27.34	-	A	*Agrostis stolonifera*	2005	L.P. Tredway	North Carolina	Wilmington

Isolate	Average CT Clade A assay	Average CT Clade B assay	Diagnosis	*Host Species*	Year Isolated	Collector	Origin	Location
NC ABR50	26.86	-	A	*Agrostis stolonifera*	2005	L.P. Tredway	North Carolina	Monroe
NC ABR51	25.22	-	A	*Agrostis stolonifera*	2005	L.P. Tredway	North Carolina	Monroe
NC ABR6	26.35	-	A	*Agrostis stolonifera*	2005	L.P. Tredway	North Carolina	Wilmington
NC ABR9	28.39	-	A	*Agrostis stolonifera*	2005	L.P. Tredway	North Carolina	Wilmington
NC-ABR14	24.35	-	A	*Poa annua*	2005	L.P. Tredway	North Carolina	Sanford
NC-ABR26	27.90	-	A	*Poa annua*	2005	L.P. Tredway	North Carolina	Blowing Rock
NC-ABR52	24.86	-	A	*Poa annua*	2005	L.P. Tredway	North Carolina	Monroe
NC-ABR52 (duplicate)	23.58	-	A	*Poa annua*	2005	L.P. Tredway	North Carolina	Monroe
NC-BR11A	23.23	-	A	*Poa annua*	2005	L.P. Tredway	North Carolina	Blowing Rock
NC-BR12A	22.37	-	A	*Poa annua*	2005	L.P. Tredway	North Carolina	Blowing Rock
NC-BR12B	21.64	-	A	*Poa annua*	2005	L.P. Tredway	North Carolina	Blowing Rock
NC-BR14A	23.61	-	A	*Poa annua*	2005	L.P. Tredway	North Carolina	Blowing Rock
NC-BR18A	26.68	-	A	*Poa annua*	2005	L.P. Tredway	North Carolina	Blowing Rock
NC-BR18A (duplicate)	24.51	-	A	*Poa annua*	2005	L.P. Tredway	North Carolina	Blowing Rock
NC-BR19A	20.79	-	A	*Poa annua*	2005	L.P. Tredway	North Carolina	Blowing Rock
NC-BR21B	20.72	-	A	*Poa annua*	2005	L.P. Tredway	North Carolina	Blowing Rock
NC-BR22B	24.53	-	A	*Poa annua*	2005	L.P. Tredway	North Carolina	Blowing Rock
NC-BR28B	23.80	-	A	*Poa annua*	2005	L.P. Tredway	North Carolina	Blowing Rock
NC-BR3B	22.93	-	A	*Poa annua*	2005	L.P. Tredway	North Carolina	Blowing Rock
NC-BR4A	25.69	-	A	*Poa annua*	2005	L.P. Tredway	North Carolina	Blowing Rock
NC-BR5B	23.62	-	A	*Poa annua*	2005	L.P. Tredway	North Carolina	Blowing Rock
NC-BR6A	23.22	-	A	*Poa annua*	2005	L.P. Tredway	North Carolina	Blowing Rock
NC-BR8A	22.55	-	A	*Poa annua*	2005	L.P. Tredway	North Carolina	Blowing Rock
NE-BI1.1-2	32.90	-	A	*Bromus inermis*	2005	J.A. Crouch	Nebraska	Red Cloud
NE-BI1.2-2	23.92	-	A	*Bromus inermis*	2005	J.A. Crouch	Nebraska	Red Cloud

Isolate	Average CT Clade A assay	Average CT Clade B assay	Diagnosis	*Host Species*	Year Isolated	Collector	Origin	Location
NE-BI1.3-5	23.47	-	A	*Bromus inermis*	2005	J.A. Crouch	Nebraska	Red Cloud
NE-F1-1.6B5	26.41	-	A	*Triticum aestivum*	2005	J.A. Crouch	Kansas	
NE-TA-F1 1.6A1	38.07	-	A	*Triticum aestivum*	2005	J.A. Crouch	Nebraska	
NE-TA-F1 1.7A2	37.86	-	A	*Triticum aestivum*	2005	J.A. Crouch	Nebraska	
NE-TA-F1 1.7C2	38.39	-	A	*Triticum aestivum*	2005	J.A. Crouch	Nebraska	
NE-TA-F1 1.7E	38.78	-	A	*Triticum aestivum*	2005	J.A. Crouch	Nebraska	
NE-TA-F1 5.12B5	37.86	-	A	*Triticum aestivum*	2005	J.A. Crouch	Nebraska	
NE-TA-F1 5.7B4	35.89	-	A	*Triticum aestivum*	2005	J.A. Crouch	Nebraska	
NE-TA-F1 5.8KS-20B-I-1	38.33	-	A	*Triticum aestivum*	2005	J.A. Crouch	Nebraska	
NE-TA-F1 6.2	37.24	-	A	*Triticum aestivum*	2005	J.A. Crouch	Nebraska	
NE-TA-F1 6.3C1	39.05	-	A	*Triticum aestivum*	2005	J.A. Crouch	Nebraska	
NE-TA-F1 6.5A3	38.55	-	A	*Triticum aestivum*	2005	J.A. Crouch	Nebraska	
NE-TA-F1 6.6B5	35.35	-	A	*Triticum aestivum*	2005	J.A. Crouch	Nebraska	
NE-TA-F1 7B4	35.44	-	A	*Triticum aestivum*	2005	J.A. Crouch	Nebraska	
NE-TA-F1 8.1A1	34.79	-	A	*Triticum aestivum*	2005	J.A. Crouch	Nebraska	
NE-TA-F16.3B5	38.84	-	A	*Triticum aestivum*	2005	J.A. Crouch	Nebraska	
NE-TA-FI 6.6KS-20B-I	30.17	-	A	*Triticum aestivum*	2005	J.A. Crouch	Nebraska	
NH-23	22.10	-	A	*Agrostis stolonifera*	1998	N. Jackson	New Hampshire	Passaconway
NJ-6340	29.18	-	A	*Poa annua*	2004	J.A. Crouch	New Jersey	
NJ-7130	25.47	-	A	*Poa annua*	2004	J.A. Crouch	New Jersey	Atlantic Co.
NJ-7130A	26.07	-	A	*Poa annua*	2004	J.A. Crouch	New Jersey	Atlantic Co.
NJ-7130B	25.10	-	A	*Poa annua*	2004	J.A. Crouch	New Jersey	Atlantic Co.
NJ-CA1-C	28.05	-	A	*Calamagrostis acutifolia*	2005	J.A. Crouch	New Jersey	Camden Co.
NJ-CA1A1	26.97	-	A	*Calamagrostis acutifolia*	2005	J.A. Crouch	New Jersey	Camden Co.
NJ-CA1A2	24.88	-	A	*Calamagrostis acutifolia*	2005	J.A. Crouch	New Jersey	Camden Co.

Isolate	Average CT Clade A assay	Average CT Clade B assay	Diagnosis	*Host Species*	Year Isolated	Collector	Origin	Location
NJ-CA1A3	28.07	-	A	*Calamagrostis acutifolia*	2005	J.A. Crouch	New Jersey	Camden Co.
NJ-CA1A4	23.87	-	A	*Calamagrostis acutifolia*	2005	J.A. Crouch	New Jersey	Camden Co.
NJ-CA1A5	28.26	-	A	*Calamagrostis acutifolia*	2005	J.A. Crouch	New Jersey	Camden Co.
NJ-CA1B3	30.26	-	A	*Calamagrostis acutifolia*	2005	J.A. Crouch	New Jersey	Camden Co.
NJ-CA1C2	24.60	-	A	*Calamagrostis acutifolia*	2005	J.A. Crouch	New Jersey	Camden Co.
NJ-CA1D1	26.90	-	A	*Calamagrostis acutifolia*	2005	J.A. Crouch	New Jersey	Camden Co.
NJ-CA1D2	26.92	-	A	*Calamagrostis acutifolia*	2005	J.A. Crouch	New Jersey	Camden Co.
NJ-CA1D3	26.64	-	A	*Calamagrostis acutifolia*	2005	J.A. Crouch	New Jersey	Camden Co.
NJ-CA1D4	27.54	-	A	*Calamagrostis acutifolia*	2005	J.A. Crouch	New Jersey	Camden Co.
NJ-CA1D5	28.35	-	A	*Calamagrostis acutifolia*	2005	J.A. Crouch	New Jersey	Camden Co.
NJ-CA1E1	26.13	-	A	*Calamagrostis acutifolia*	2005	J.A. Crouch	New Jersey	Camden Co.
NJ-CA1E2	24.51	-	A	*Calamagrostis acutifolia*	2005	J.A. Crouch	New Jersey	Camden Co.
NJ-CA1E3	26.56	-	A	*Calamagrostis acutifolia*	2005	J.A. Crouch	New Jersey	Camden Co.
NJ-CA1E4	26.13	-	A	*Calamagrostis acutifolia*	2005	J.A. Crouch	New Jersey	Camden Co.
NJ-CA1G2	28.88	-	A	*Calamagrostis acutifolia*	2005	J.A. Crouch	New Jersey	Camden Co.
NJ-CA1G3	25.05	-	A	*Calamagrostis acutifolia*	2005	J.A. Crouch	New Jersey	Camden Co.
NJ-CA1L5	25.66	-	A	*Calamagrostis acutifolia*	2005	J.A. Crouch	New Jersey	Camden Co.
NJ-CA1L5 (duplicate)	26.03	-	A	*Calamagrostis acutifolia*	2005	J.A. Crouch	New Jersey	Camden Co.
NJ-CA1N2	34.51	-	A	*Calamagrostis acutifolia*	2005	J.A. Crouch	New Jersey	Camden Co.
NJ-CA1N3	23.80	-	A	*Calamagrostis acutifolia*	2005	J.A. Crouch	New Jersey	Camden Co.
NJ-CA1N4	27.57	-	A	*Calamagrostis acutifolia*	2005	J.A. Crouch	New Jersey	Camden Co.

Isolate	Average CT Clade A assay	Average CT Clade B assay	Diagnosis	*Host Species*	Year Isolated	Collector	Origin	Location
NJ-CA1N5	27.65	-	A	*Calamagrostis acutifolia*	2005	J.A. Crouch	New Jersey	Camden Co.
NJ-CAIB3	25.39	-	A	*Calamagrostis acutifolia*	2005	J.A. Crouch	New Jersey	Camden Co.
NJ-CAIL3	26.74	-	A	*Calamagrostis acutifolia*	2005	J.A. Crouch	New Jersey	Camden Co.
NJ-DG4-5	37.21	-	A	*Dactylis glomerata*	2004	J.A. Crouch	New Jersey	New Brunswick
NJ-HF2-17A	25.20	-	A	*Poa annua*	2004	J.A. Crouch	New Jersey	New Brunswick
NJ-RWCC	19.23	-	A	*Poa annua*	2004	J.A. Crouch	New Jersey	Ridgewood
NY-8422	22.21	-	A	*Poa annua*	2004	J.A. Crouch	New York	Scarsdale
NY-8422 (duplicate)	28.73	-	A	*Poa annua*	2006	J.A. Crouch	New York	Scarsdale
NY-8893	28.17	-	A	*Agrostis stolonifera*	2006	J.A. Crouch	New York	
OW15	24.78	-	A	*Agrostis stolonifera*	2006	M. Tomaso-Peterson	Mississippi	West Point
OW15 A2-3	27.93	-	A	*Agrostis stolonifera*	2006	M. Tomaso-Peterson	Mississippi	West Point
OW15 B4-1	27.75	-	A	*Agrostis stolonifera*	2006	M. Tomaso-Peterson	Mississippi	West Point
OW15 B4-1	26.22	-	A	*Agrostis stolonifera*	2006	M. Tomaso-Peterson	Mississippi	West Point
OW15 T4-1	25.05	-	A	*Agrostis stolonifera*	2006	M. Tomaso-Peterson	Mississippi	West Point
OW15H3-2	23.37	-	A	*Agrostis stolonifera*	2006	M. Tomaso-Peterson	Mississippi	West Point
PA-5001-4	33.74	-	A	*Poa annua*	2000	W. Uddin	Pennsylvania	Malvern
PA-5010-1	27.46	-	A	*Poa annua*	2000	W. Uddin	Pennsylvania	Mount Union
PA-5011-1	25.83	-	A	*Poa annua*	2000	W. Uddin	Pennsylvania	Royersford
PA-5011-4	27.16	-	A	*Poa annua*	2000	W. Uddin	Pennsylvania	Royersford
PA-5018-1	34.55	-	A	*Poa annua*	2000	W. Uddin	Pennsylvania	Royersford
PA-VALP-01	34.04	-	A	*Poa annua*	2000	W. Uddin	Pennsylvania	University Park
PA-VALP-02	27.05	-	A	*Poa annua*	2000	W. Uddin	Pennsylvania	University Park
PA-WH-3	27.66	-	A	*Poa annua*	2000	W. Uddin	Pennsylvania	Leesport
PA-WH-4	27.44	-	A	*Poa annua*	2000	W. Uddin	Pennsylvania	Leesport
PA1	29.75	-	A	Agrostis stolonifera	2005	L.P. Tredway	Virginia	Virginia Beach

Isolate	Average CT Clade A assay	Average CT Clade B assay	Diagnosis	*Host Species*	Year Isolated	Collector	Origin	Location
PA2	24.55	-	A	Agrostis stolonifera	2005	L.P. Tredway	Virginia	Virginia Beach
PA4	25.39	-	A	Agrostis stolonifera	2005	L.P. Tredway	Virginia	Virginia Beach
PA5	29.35	-	A	Agrostis stolonifera	2005	L.P. Tredway	Virginia	Virginia Beach
RI-22	23.37	-	A	*Agrostis stolonifera*	1998	N. Jackson	Rhode Island	Metacomet
RI-8	29.77	-	A	*Poa annua*	1998	N. Jackson	Rhode Island	Washington Co.
RI-9	28.37	-	A	*Poa annua*	1998	N. Jackson	Rhode Island	Wakefield
SC44	24.47	-	A	*Poa annua*	2003	F.P. Wong	California	San Jose
SC9	26.68	-	A	*Poa annua*	2003	F.P. Wong	California	San Jose
SCC1	26.11	-	A	*Poa annua*	2003	F.P. Wong	California	San Jose
SCI	26.17	-	A	*Poa annua*	2003	F.P. Wong	California	San Jose
SH22	28.22	-	A	*Poa annua*	2003	F.P. Wong	California	San Bernadino
SH22 (duplicate)	27.02	-	A	*Poa annua*	2003	F.P. Wong	California	San Bernadino
SH27	34.73	-	A	*Poa annua*	2003	F.P. Wong	California	San Bernadino
SH29	24.47	-	A	*Poa annua*	2003	F.P. Wong	California	San Bernadino
TCGC 5-21	29.60	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
TCGC 5-21 (duplicate)	26.37	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
TCGC 5-23	29.27	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
TCGC 5-26	28.40	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
TCGC 5-28	29.68	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
TCGC 5-29	29.75	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
TCGC 5-34	26.46	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
TCGC 5-35	29.03	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
TCGC 5-37	33.41	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
TCGC 5-38	27.79	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
TCGC 5-39	28.41	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
TCGC 5-42	29.94	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula

Isolate	Average CT Clade A assay	Average CT Clade B assay	Diagnosis	*Host Species*	Year Isolated	Collector	Origin	Location
TCGC 5-45	26.61	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
TCGC 5-47	29.32	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
TCGC 5-5	29.31	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
TCGC 5-52	28.96	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
TCGC 5-53	27.20	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
TCGC 5-56	29.97	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
TCGC 5-60	28.54	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
TCGC 5-61	27.98	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
TCGC 5-62	30.57	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
TCGC 5-62 (duplicate)	31.46	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
TCGC 5-64	26.04	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
TCGC 5-65	29.51	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
TCGC 5-67	28.71	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
TCGC 5-68	34.43	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
TCGC 5-75	26.01	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
TCGC 6-66	33.28	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
TCGC 8-10	31.07	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
TCGC 8-11	30.41	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
TCGC 8-12	32.16	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
TCGC 8-6	28.52	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
TCGC 8-6 (duplicate)	26.48	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
TCGC 8-7	31.70	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
TCGC 8-7 (duplicate)	31.91	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
TCGC 8-8	29.75	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
TCGC 8-9	31.07	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula

Isolate	Average CT Clade A assay	Average CT Clade B assay	Diagnosis	*Host Species*	Year Isolated	Collector	Origin	Location
TN-GBGC3	22.91	-	A	*Agrostis stolonifera*		L.P. Tredway	Tennessee	
TRACY 1-3	28.96	-	A	*Poa annua*		F.P. Wong	California	
TRACY 2-1	28.95	-	A	*Poa annua*		F.P. Wong	California	
TX-26	22.80	-	A	*Agrostis stolonifera*	1998	N. Jackson	Texas	Sugartree
NE-TA-F1 6.3 C5	36.76	39.31	A[a]	*Triticum aestivum*	2005	J.A. Crouch	Nebraska	
00110	-	25.52	B	*Agrostis stolonifera*	2000	T. Hsiang	Canada	Milton, ON
00114	-	27.76	B	*Agrostis stolonifera*	2000	T. Hsiang	Canada	Milton, ON
00121	-	26.99	B	*Agrostis stolonifera*	2000	T. Hsiang	Canada	Osprey Valley, ON
00124	-	27.33	B	*Agrostis stolonifera*	2000	T. Hsiang	Canada	Osprey Valley, ON
00126	-	24.10	B	*Agrostis stolonifera*	2000	T. Hsiang	Canada	Osprey Valley, ON
00127	-	25.16	B	*Agrostis stolonifera*	2000	T. Hsiang	Canada	Osprey Valley, ON
00128	-	28.83	B	*Agrostis stolonifera*	2000	T. Hsiang	Canada	Osprey Valley, ON
00132	-	24.02	B	*Agrostis stolonifera*	2000	T. Hsiang	Canada	Osprey Valley, ON
00133	-	27.51	B	*Agrostis stolonifera*	2000	T. Hsiang	Canada	Osprey Valley, ON
00133	-	25.78	B	*Agrostis stolonifera*	2000	T. Hsiang	Canada	Osprey Valley, ON
00134	-	28.57	B	*Agrostis stolonifera*	2000	T. Hsiang	Canada	Osprey Valley, ON
00137	-	25.29	B	*Agrostis stolonifera*	2000	T. Hsiang	Canada	Osprey Valley, ON
00143	-	27.06	B	*Agrostis stolonifera*	2000	T. Hsiang	Canada	Osprey Valley, ON
00145	-	25.63	B	*Agrostis stolonifera*	2000	T. Hsiang	Canada	Osprey Valley, ON
00145	-	27.44	B	*Agrostis stolonifera*	2000	T. Hsiang	Canada	Osprey Valley, ON
00147	-	26.04	B	*Agrostis stolonifera*	2000	T. Hsiang	Canada	Osprey Valley, ON
00149	-	29.34	B	*Agrostis stolonifera*	2000	T. Hsiang	Canada	Osprey Valley, ON
00151	-	25.29	B	*Agrostis stolonifera*	2000	T. Hsiang	Canada	Osprey Valley, ON
00156	-	27.51	B	*Agrostis stolonifera*	2000	T. Hsiang	Canada	Osprey Valley, ON

Isolate	Average CT Clade A assay	Average CT Clade B assay	Diagnosis	*Host Species*	Year Isolated	Collector	Origin	Location
00158	-	25.65	B	*Agrostis stolonifera*	2000	T. Hsiang	Canada	Osprey Valley, ON
00161	-	26.14	B	*Agrostis stolonifera*	2000	T. Hsiang	Canada	Osprey Valley, ON
00162	-	25.30	B	*Agrostis stolonifera*	2000	T. Hsiang	Canada	Osprey Valley, ON
00163	-	25.81	B	*Agrostis stolonifera*	2000	T. Hsiang	Canada	Osprey Valley, ON
00185	-	25.83	B	*Agrostis stolonifera*	2000	T. Hsiang	Canada	Downsview, ON
99003	-	33.39	B	*Agrostis stolonifera*	1999	T. Hsiang	Pennsylvania	College Park
99325	-	28.17	B	*Poa pratensis*	1999	T. Hsiang	Canada	Lacombe, Alberta
279CGCT5	-	28.26	B	*Agrostis stolonifera*	2006	J.E. Kaminski	Connecticut	
CBS 303.69	-	28.29	B	*Agrostis tenuis*	1967	CBS 303.69	Germany	
CBS 303.69 (duplicate)	-	27.94	B	*Agrostis tenuis*	1967	CBS 303.69	Germany	
CBS 304.69	-	28.72	B	*Ammophila arenaria*	1967	CBS 304.69	Germany	
CT-18	-	27.54	B	*Agrostis stolonifera*	1998	N. Jackson	Connecticut	Farmington
CT-27	-	25.10	B	*Agrostis stolonifera*	1998	N. Jackson	Connecticut	Wethersfield
CT-4	-	36.44	B	*Poa annua*	1998	N. Jackson	Connecticut	Quaker Ridge
D8237	-	25.57	B	*Poa annua*		J.A. Crouch	Pennsylvania	Ambler
DGA5	-	26.16	B	*Dactylis glomerata*	2005	J.A. Crouch	New Jersey	
DGI1	-	26.83	B	*Dactylis glomerata*	2005	J.A. Crouch	New Jersey	
DGI10	-	26.11	B	*Dactylis glomerata*	2005	J.A. Crouch	New Jersey	
DGI11	-	25.11	B	*Dactylis glomerata*	2005	J.A. Crouch	New Jersey	
DGI13	-	30.08	B	*Dactylis glomerata*	2005	J.A. Crouch	New Jersey	
RI-1	-	27.54	B	*Poa annua*	1998	N. Jackson	Rhode Island	Alpine
CT-17	-	27.53	B	*Agrostis stolonifera*	1998	N. Jackson	Connecticut	Farmington
MA-20	-	27.70	B	*Agrostis stolonifera*	1998	N. Jackson	Massachusetts	Ipswich
MA-24	-	25.58	B	*Agrostis stolonifera*	1998	N. Jackson	Massachusetts	Pine Acres
MA-28	-	28.24	B	*Agrostis canina*	1998	N. Jackson	Massachusetts	Woodbridge
MA-29	-	26.03	B	*Poa annua*	1998	N. Jackson	Massachusetts	Cummaquid

Isolate	Average CT Clade A assay	Average CT Clade B assay	Diagnosis	*Host Species*	Year Isolated	Collector	Origin	Location
MA-5	-	26.62	B	*Poa annua*	1998	N. Jackson	Massachusetts	Quashnett Valley
MA-6	-	28.94	B	*Poa annua*	1998	N. Jackson	Massachusetts	Salem
MA-6772	-	28.55	B	*Poa annua*	2005	J.A. Crouch	Massachusetts	
NC-ABR27	-	27.86	B	*Poa annua*	2005	L.P. Tredway	North Carolina	Blowing Rock
NC-ABR48	-	25.46	B	*Agrostis stolonifera*	2005	L.P. Tredway	North Carolina	Monroe
NC-BR27B	-	29.11	B	*Poa annua*	2005	L.P. Tredway	North Carolina	Blowing Rock
NJ WKDG2A22	-	24.57	B	*Dactylis glomerata*	2005	J.A. Crouch	New Jersey	
NJ WKDG2A24	-	30.14	B	*Dactylis glomerata*	2005	J.A. Crouch	New Jersey	
NJ-4990	-	26.85	B	*Poa annua*	2004	J.A. Crouch	New Jersey	
NJ-6553	-	29.01	B	*Poa annua*	2004	J.A. Crouch	New Jersey	
NJ-6607	-	26.89	B	*Poa annua*	2004	J.A. Crouch	New Jersey	
NJ-DG4-4	-	27.73	B	*Dactylis glomerata*	2004	J.A. Crouch	New Jersey	New Brunswick
NJ6553	-	26.93	B	*Poa annua*	2004	J.A. Crouch	New Jersey	
NY-16	-	30.64	B	*Agrostis stolonifera*	1998	N. Jackson	New York	Back O'Beyond
NY-7	-	29.10	B	*Poa annua*	1998	N. Jackson	New York	Sands Point
PA-50002	-	28.63	B	*Poa annua*	2000	W. Uddin	Pennsylvania	Bernville
PA-50002 (duplicate)	-	28.23	B	*Poa annua*	2000	W. Uddin	Pennsylvania	Bernville
PA-50005	-	27.38	B	*Poa annua*	2000	W. Uddin	Pennsylvania	Bernville
PA-50005 (duplicate)	-	28.31	B	*Poa annua*	2000	W. Uddin	Pennsylvania	Bernville
PA-50181	-	30.76	B	*Poa annua*	2000	W. Uddin	Pennsylvania	Reedsville
PA-50623	-	29.18	B	*Poa annua*	2000	W. Uddin	Pennsylvania	Reedsville
PA-50621	-	25.33	B	*Poa annua*	2000	W. Uddin	Pennsylvania	Farmington
PA-ANB4410	-	24.97	B	*Poa annua*	2000	W. Uddin	Pennsylvania	Bally
PA-ANB4410 (duplicate)	-	27.43	B	*Poa annua*	2000	W. Uddin	Pennsylvania	Bally
PA-S1112	-	27.40	B	*Poa annua*	2000	W. Uddin	Pennsylvania	Bethlehem

Isolate	Average CT Clade A assay	Average CT Clade B assay	Diagnosis	*Host Species*	Year Isolated	Collector	Origin	Location
PA-S2113	-	30.87	B	*Poa annua*	2000	W. Uddin	Pennsylvania	
RI-10	-	27.49	B	*Poa annua*	1998	N. Jackson	Rhode Island	Wannamoisett
TCGC 5-70	-	28.07	B	*Poa annua*	2002	F.P. Wong	California	Temecula
PA-50183	25.92	24.99	both[b]	*Poa annua*	2000	W. Uddin	Pennsylvania	Reedsville
KS-DGI12	-	-	No Ct[c]	*Dactylis glomerata*	2005	J.A. Crouch	New Jersey	

[a]Isolate was diagnosed as belonging to clade A following visual inspection of amplification curves. Low fluorescence intensity and late CT values (>40.0) was observed for this sample when tested using the B-Apn2 assay.
[b]Isolate PA-50183 produced a positive diagnosis from both A-Apn2 and B-Apn2 assays, consistent with RFLP fingerprint data (Crouch et al. 2008) previously generated for this isolate
[c]No diagnosis could be made using either assay. Analysis with the NanoDrop spectrophotometer showed an overabundance of compounds at A230.

Supplemental Table S2. Samples of non-target fungal species used as negative controls for *Colletotrichum cereale* real-time PCR assays. CT=cycle threshold.

Isolate		Host Species	Average Ct A	Average Ct B	Diagnosis
Cryphonectria parasitica	EP155	*Castanea dentata*	–	–	No Ct
Magnaporthe oryzae	7015	pure culture	–	–	No Ct
Phytophthora infestans	PI1	*Solanum tuberosum*	–	–	No Ct
Sclerotinia homoeocarpa	HF218_10	*Agrostis stolonifera*	–	–	No Ct
Colletotrichum graminicola	M1.001	*Zea mays*	–	–	No Ct
Colletotrichum sublineola	S3.001	*Sorghum bicolor*	–	–	No Ct
Colletotrichum navitas	CBS125086	*Panicum virgatum*	–	–	No Ct
Colletotrichum falcatum	MAFF306299	*Saccharum officinarum*	–	–	No Ct
Colletotrichum hanaui	MAFF305404	*Digitaria ciliaris*	–	–	No Ct
Colletotrichum nicholsonii	MAFF305428	*Paspalum dilatatum*	–	–	No Ct

Supplemental Table S3. Summary of biological Replicates of Cultured Samples of *Colletotrichum cereale* tested to determine clade membership (A or B) using real-time PCR assays. CT=cycle threshold.

Isolate	Average CT Clade A assay	Average CT Clade B assay	Diagnosis	Host Species	Year Isolated	Collector	Origin	Location
1050-AC	25.05	-	A	*Aegilops cylindrica*	1985		Arkansas	Washington Co.
1050AC (duplicate)	26.35	-	A	*Aegilops cylindrica*	1985		Arkansas	Washington Co.
avg/std dev.	***25.70***	***0.92***						
AHCC 10-60	27.10	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 10-60 (duplicate)	28.71	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
avg/std dev.	***27.91***	***1.14***						
AHCC 80	28.43	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 80 (duplicate)	25.55	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
avg/std dev.	***26.99***	***2.04***						
AHCC 81	28.37	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
AHCC 81 (duplicate)	26.57	-	A	*Poa annua*	2005	F.P. Wong	California	Arcadia
avg/std dev.	***27.47***	***1.27***						
ANCG17-15	23.57	-	A	*Poa annua*	2004	F.P. Wong	California	Pasadena
ANCG17-15 (duplicate)	24.11	-	A	*Poa annua*	2004	F.P. Wong	California	Pasadena
avg/std dev.	***23.84***	***0.38***						
CBS 148.34	23.49	-	A	*Avena*	1934	CBS 148.34	Canada	Alberta

Isolate	Average CT Clade A assay	Average CT Clade B assay	Diagnosis	Host Species	Year Isolated	Collector	Origin	Location
				sativa				
CBS 148.34 (duplicate)	24.83	-	A	*Avena sativa*	1934	CBS 148.34	Canada	Alberta
avg/std dev.	***24.16***	***0.95***						
CBS 240.49	31.66	-	A	*Avena sativa*	1949	CBS 240.49	Germany	
CBS 240.49 (duplicate)	22.45	-	A	*Avena sativa*	1949	CBS 240.49	Germany	
avg/std dev.	***27.06***	***6.51***						
CT-2	22.03	-	A	*Poa annua*		N. Jackson	Connecticut	
CT-2 (duplicate)	28.41	-	A	*Poa annua*			Connecticut	
avg/std dev.	***25.22***	***4.51***						
KS-10-EC2C1	25.12	-	A	*Elymus canadensis*	2005	J.A. Crouch	Kansas	Manhattan
KS-10-EC2C2 (duplicate)	25.21	-	A	*Elymus canadensis*	2005	J.A. Crouch	Kansas	Manhattan
avg/std dev.	***25.17***	***0.06***						
KS-10-EC3E2	23.73	-	A	*Elymus canadensis*	2005	J.A. Crouch	Kansas	Manhattan
KS-10-EC3E2 (duplicate)	24.13	-	A	*Elymus canadensis*	2005	J.A. Crouch	Kansas	Manhattan
avg/std dev.	***23.93***	***0.28***						
KS-20-DGD5	26.29	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGD5 (duplicate)	25.34	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan

Isolate	Average CT Clade A assay	Average CT Clade B assay	Diagnosis	Host Species	Year Isolated	Collector	Origin	Location
avg/std dev.	***25.82***	***0.67***						
KS-20-DGV2	25.82	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-DGV2 (duplicate)	25.66	-	A	*Dactylis glomerata*	2005	J.A. Crouch	Kansas	Manhattan
avg/std dev.	***25.74***	***0.11***						
KS-20-EVD2	26.28	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVD2 (duplicate)	35.46	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
avg/std dev.	***30.87***	***6.49***						
KS-20-EVV1	25.54	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
KS-20-EVV1 (duplicate)	25.82	-	A	*Elymus virginicus*	2005	J.A. Crouch	Kansas	Manhattan
avg/std dev.	***25.68***	***0.20***						
KS-FE7A4	25.69	-	A	*Festuca elatior*	2005	J.A. Crouch	Kansas	Manahattan
KS-FE7A4 (duplicate)	22.70	-	A	*Festuca elatior*	2005	J.A. Crouch	Kansas	Manahattan
avg/std dev.	***24.20***	***2.11***						
KS-TA-F9 W1.2-4	31.58	-	A	*Triticum aestivum*	2005	J.A. Crouch	Kansas	
KS-TA-F9 W4-2.4 (duplicate)	30.33	-	A	*Triticum aestivum*	2005	J.A. Crouch	Kansas	
avg/std dev.	***30.96***	***0.88***						

Isolate	Average CT Clade A assay	Average CT Clade B assay	Diagnosis	Host Species	Year Isolated	Collector	Origin	Location
MA-11	21.09	-	A	*Poa annua*		N. Jackson	Massachusetts	
MA-11 (duplicate)	21.07	-	A	*Poa annua*		N. Jackson	Massachusetts	
avg/std dev.	***21.08***	***0.01***						
MAFF305427	27.10	-	A	*Avena sativa*			Japan	Kumamota Prefecture
MAFF305427 (duplicate)	23.62	-	A	*Avena sativa*			Japan	Kumamota Prefecture
avg/std dev.	***25.36***	***2.46***						
MAFF305436	23.73	-	A	*Dactylis glomerata*			Japan	Tochigi Prefecture
MAFF305436 (duplicate)	20.72	-	A	*Dactylis glomerata*			Japan	Tochigi Prefecture
MAFF305436 (duplicate)	25.23	-	A	*Dactylis glomerata*			Japan	Tochigi Prefecture
avg/std dev.	***22.98***	***3.19***						
NBR-13	24.32	-	A	*Poa annua*		N. Jackson	Canada	New Brunswick
NBR-13 (duplicate)	25.66	-	A	*Poa annua*		N. Jackson	Canada	New Brunswick
avg/std dev.	***24.99***	***0.95***						
NC-ABR52	24.86	-	A	*Poa annua*	2005	L.P. Tredway	North Carolina	Monroe
NC-ABR52 (duplicate)	23.58	-	A	*Poa annua*	2005	L.P. Tredway	North Carolina	Monroe
avg/std dev.	***24.22***	***0.91***						
NC-BR18A	26.68	-	A	*Poa annua*			North Carolina	
NC-BR18A (duplicate)	24.51	-	A	*Poa annua*			North Carolina	

Isolate	Average CT Clade A assay	Average CT Clade B assay	Diagnosis	Host Species	Year Isolated	Collector	Origin	Location
avg/std dev.	***25.60***	***1.53***						
NJ-CA1L5	25.66	-	A	*Calamagrostis acutifolia*	2005	J.A. Crouch	New Jersey	Camden Co.
NJ-CA1L5 (duplicate)	26.03	-	A	*Calamagrostis acutifolia*	2005	J.A. Crouch	New Jersey	Camden Co.
avg/std dev.	***25.85***	***0.26***						
NY-8422	22.21	-	A	*Poa annua*	2004	J.A. Crouch	New York	Scarsdale
NY-8422 (duplicate)	28.73	-	A	*Poa annua*	2006	J.A. Crouch	New York	Scarsdale
avg/std dev.	***25.47***	***4.61***						
SH22	28.22	-	A	*Poa annua*	2003	F.P. Wong	California	San Bernadino
SH22 (duplicate)	27.02	-	A	*Poa annua*	2003	F.P. Wong	California	San Bernadino
avg/std dev.	***27.62***	***0.85***						
TCGC 5-21	29.60	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
TCGC 5-21 (duplicate)	26.37	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
avg/std dev.	***27.99***	***2.28***						
TCGC 5-62	30.57	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
TCGC 5-62 (duplicate)	31.46	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
avg/std dev.	***31.02***	***0.63***						

Isolate	Average CT Clade A assay	Average CT Clade B assay	Diagnosis	Host Species	Year Isolated	Collector	Origin	Location
TCGC 8-6	28.52	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
TCGC 8-6 (duplicate)	26.48	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
avg/std dev.	***27.50***	***1.44***						
TCGC 8-7	31.70	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
TCGC 8-7 (duplicate)	31.91	-	A	*Poa annua*	2002	F.P. Wong	California	Temecula
avg/std dev.	***31.81***	***0.15***						
CBS 303.69	-	28.29	B	*Agrostis tenuis*	1969	CBS 303.69	Germany	
CBS 303.69 (duplicate)	-	27.94	B	*Agrostis tenuis*	1969	CBS 303.69	Germany	
avg/std dev.	***27.94***							
PA-5000-2	-	28.63	B	*Poa annua*		W. Uddin	Pennsylvania	Bernville, PA
PA-5000-2 (duplicate)	-	28.23	B	*Poa annua*		W. Uddin	Pennsylvania	Bernville, PA
avg/std dev.	***28.43***	***0.28***						
PA-50005	-	27.38	B	*Poa annua*		W. Uddin	Pennsylvania	Bernville, PA
PA-50005 (duplicate)	-	28.31	B	*Poa annua*		W. Uddin	Pennsylvania	Bernville, PA
avg/std dev.	***27.85***	***0.66***						
PA-ANB4410	-	24.97	B	*Poa annua*		W. Uddin	Pennsylvania	Bally, PA
PA-ANB4410 (duplicate)	-	27.43	B	*Poa annua*		W. Uddin	Pennsylvania	Bally, PA
avg/std dev.	***26.20***	***1.74***						

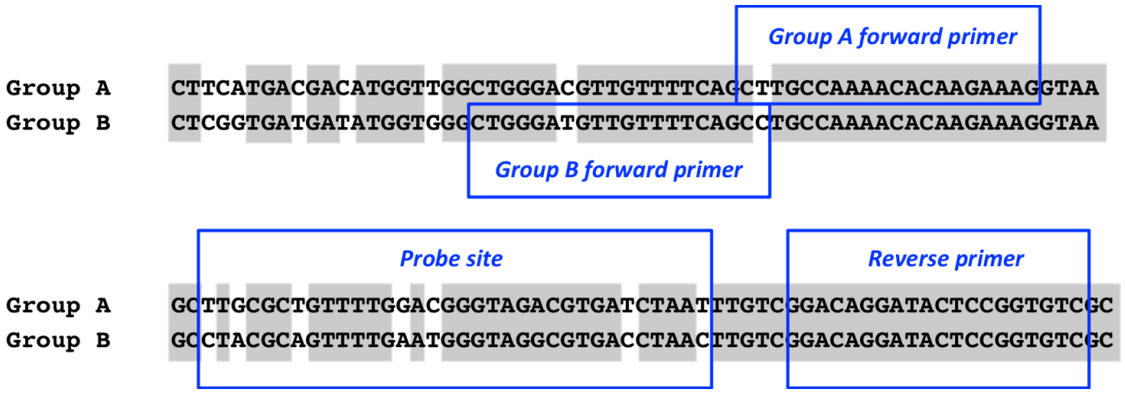

Figure 1. Placement of primers and probes used in real-time PCR. Part of the DNA lyase (*Apn2*) nucleotide sequence used as the template for real-time PCR assay development. Shown is the placement of *Colletotrichum cereale* group-specific forward PCR primers A-Apn2-F, B-Apn2-F with probe sites for *C. cereale* clade A probe A-Apn2 and *C. cereale* clade B probe B-Apn2. Both sets of forward primers and probes utilized the same reverse primer, Apn2-R. Species-specific SNPs that differentiate target lineages are denoted by grey and white shading. Probe binding sites differed between the two clades by seven SNPs

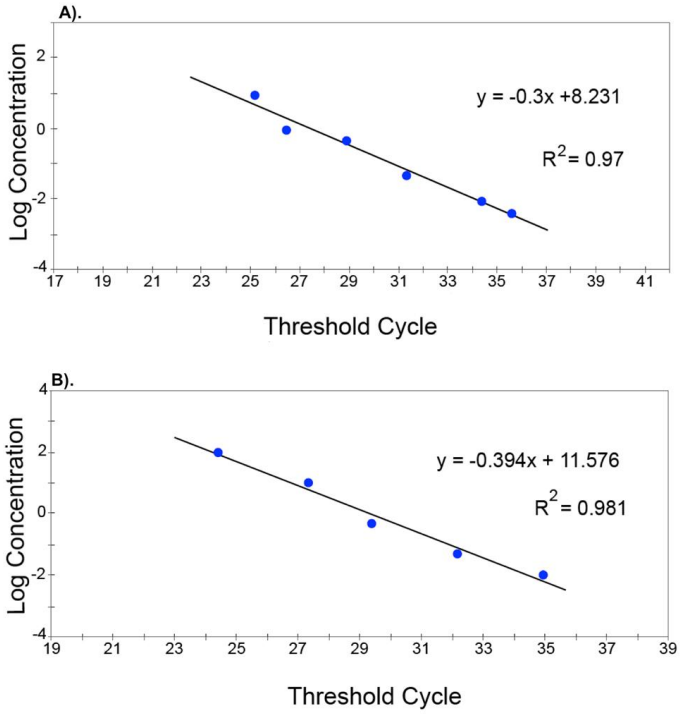

Figure 2. Real-time PCR standard curves. Real-time PCR standard curves showing the linear relationship between the log of known DNA concentrations and the second derivative cycle threshold (C_T) value. C_T values represent positive samples when the fluorescent signal crosses the amplification threshold prior to cycle 40. (a). *C. cereale* clade A probe A-Apn2 with lower detection limit of 4 pg; and (b). *C. cereale* clade B probe B-Apn2, with lower detection limit of 5 pg

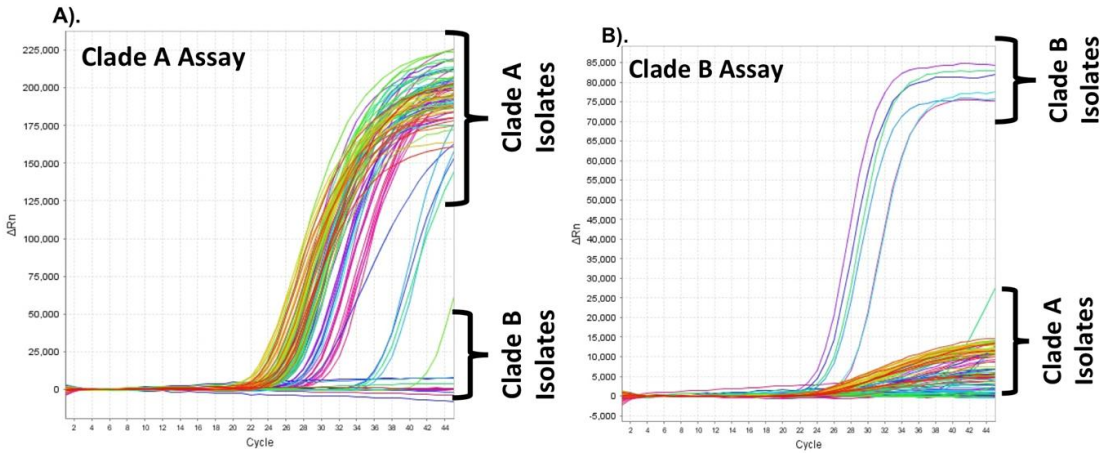

Figure 3. Real-time PCR amplification plots. Real-time PCR amplification plots of *Colletotrichum cereale* probes used to screen a 96-well plate of *C. cereale* infected samples. Negative controls are labeled. Positive controls are included within samples diagnosed as belonging to clade A or B. (a). *C. cereale* clade A probe A-Apn2; (b). *C. cereale* clade B probe B-Apn2

CHAPTER 2: Analysis of Microbial Communities in the Soil of Annual Bluegrass Putting Green Turf Highlights the Importance of Seasonality

ABSTRACT

There is increasing interest in understanding plant-associated microbial communities and how these microorganisms might be utilized to improve plant health. However, little is known about the microbial communities resident in the turfgrass ecosystem, and if these communities vary over time. The objectives of this study were to perform a community-wide inventory of the archaea, bacteria and fungi that inhabit the soil of annual bluegrass (*Poa annua*) putting green turf throughout a growing season, and from these data, to determine the core microbiota inhabiting this environment. Soil was sampled five times throughout the year (June 2014, July 2014, August 2014, April 2015, June 2015) from turfgrass plots receiving five different rates of nitrogen (no nitrogen, 4.9 kg N ha^{-1} every 7, 14, or 28 days, and 9.8 kg N ha^{-1} every 7 days). The nuclear ribosomal DNA 16s and ITS regions were sequenced using the Illumina MiSeq platform, generating 3.84 X 10^7 reads, representing 1.5 X 10^5 operational taxonomic units (OTUs). In total, 17 archaeal taxa were identified at the species level, 53% of which were members of the Crenarchaeota, a phylum largely composed of the ammonia-oxidizing archaea clone SAGMA-X. Proteobacteria was the most abundant bacterial phylum, comprising 36% of the 442 taxa identified. Within this phylum, genera including *Brevibacterium*, *Burkholderia* and *Pseudomonas*, known to possess antagonistic activity against other microorganisms, were identified. Members of the Ascomycota represented 27% of the 11 fungal taxa identified at the genus level. Fungi in the Glomeromycota, predicted to be members of mycrorhizal order Paraglomerales, were also widespread in the soil, but in

low abundance. Microbial alpha diversity was high in all samples, but there was a significant difference between diversity metrics for archaea/bacteria on two sampling dates, where samples collected from June 2015 exhibited significantly higher archaea/bacteria diversity than samples collected in June 2014 and April 2015. Distance trees derived from Bray Curtis matrices revealed clustering by sample date for all microbial populations. Analysis of similarities (ANOSIM) supported these groupings, suggesting that seasonality can influence community dynamics in annual bluegrass putting green turf. A core microbiome consisting of one archaeal OTU, twenty-seven bacterial OTUs, and one fungal OTU was identified from all samples. This inventory of the turfgrass soil microbiome highlights the wide array of microorganisms in this system and may prove useful for future investigations seeking to harness microorganisms that may influence turfgrass health. It may also help researchers who seek to better understand the functional and biological role of core microbial communities in the soil of annual bluegrass putting green turf.

INTRODUCTION

Soil microorganisms are vital constituents of all ecosystems and often contribute to important biological and chemical processes. In agricultural systems, rhizosphere microbes are essential for maintaining plant productivity, nutrient release, and suppression of plant pathogenic microorganisms (Arias et al. 2005, van der Heijden et al. 2008, Mendes et al. 2011). However, the population and community structure of microbes in the rhizosphere of agricultural soils are known to respond to changes in the environment (Fleissbaach and Mader 2004, van Diepeningen et al. 2006). There has been considerable interest in examining how microbial communities react to anthropogenic inputs to the environment, and whether these inputs might be altered to select for healthy microbial populations that promote sustainability, reduce the need for fertilizer and chemical inputs, and increase plant yield in agricultural settings (Bakker et al. 2012).

To date, research examining microbial communities in agriculture, how they are impacted by chemical inputs, and the role they play in contributing to healthy plants has largely been limited to major agricultural systems that have significant global economic impact (see Gosling et al. 2006, Perez-Piqueres et al. 2006, Fliessbach et al. 2009). In contrast, little is known about the microbial constituents within the ecosystems of specialty horticultural crops grown for fruit, flowers or vegetable production, or plants cultivated as components of the aesthetic environment such as turfgrass and landscape plants. In the United States alone, turfgrass encompasses at least 20.2 million hectares (50 million acres; National Turfgrass Federation 2009), representing an industry with an estimated value of $40 billion annually (National Turfgrass Federation 2009). Of this land area, golf courses represent a small fraction (no more than 5% of the total turf cover

in any state) of turfgrass sites in the United States (Breuninger et al. 2013), but generate significant revenue (National Turfgrass Federation 2009). For example, in 1999, golf course revenue was $573 million in New Jersey alone (Govindasamy et al. 2007).

Since the early 1990s, organizations such as the United States Golf Association, the Royal Canadian Golf Association, and the European Golf Association have actively promoted environmental stewardship and the reduction of environmental impacts associated with golf and the construction of new courses (Wheeler and Naughright 2006). There is increasing evidence that golf courses promote biodiversity, and can maintain essential habitats for flora and fauna. For example, Colding and Folke (2009) found that golf courses possess significantly more ecological value (measured as wildlife species diversity, abundance, occurrence, and reproductive success) compared to agricultural and urban ecosystems. In fact, over half of the golf courses studied had ecological value greater than or equal to preserved natural areas (Colding and Folke 2009), a finding that is largely due to the ability of golf courses to support threatened species and preserve rare habitats (Terman 1997, Colding et al. 2009, Simmons and Jarvie 2001). For example, a vulnerable species of newt, *Triturus cristatus*, was only found in golf course ponds during a survey in Sweden (Colding et al. 2009), while the Royal St. George's Golf Course in the United Kingdom was documented to host multiple species of wild orchids, including one extremely rare orchid species (Simmons and Jarvie 2001, Gange et al. 2003). In terms of habitat, golf courses can support diminishing vegetated sites, such as riparian systems (Merola-Zwartjes and DeLong 2005) and forested ecosystems (Heuberger and Putz 2003), areas that can provide vital habitat for amphibians (Mackey et al. 2014) and native or threatened avian species (Rodewald et al. 2005). While these

studies provide important information regarding the ability of golf courses to support larger plant and animal species, they provide no insight about microorganisms in the soil and their contributions to the function of this unique ecosystem.

The thatch layer (organic layer of living and dead plant material between soil surface and aboveground vegetation) in creeping bentgrass (*Agrostis stolonifera* L.) putting green turf has been shown to contain more bacteria, fungi and actinomycetes than soil at lower depths, and that the microbial counts in these locations were similar to those observed in native soils (Mancino et al. 1993). However, when compared to less intensely managed sites such as fairways and roughs, annual bluegrass (*Poa annua*) putting greens were found to contain fewer microbes (reported as phospholipid fatty acid profiles), suggesting the aggressive management practices commonly employed on putting greens may negatively impact resident microbial community (Bartlett et al. 2008). The resident microbial community is often defined as the core microbiome, referring to the microorganisms that are consistently recovered from a specific habitat (Turnbaugh *et al*., 2007, Hamady and Knight 2009). Core microbial communities are frequently associated with critical roles in the function of that environment (Shade and Handelsman 2012). To date, a core microbiome has not been identified from cultivated turfgrass hosts; however, grazed pastures in the European Alps have been shown to have a more homogeneous microbial core community across regions compared to native grasslands, suggesting that land use may be an important driver of such communities (Geremia et al. 2015). As such, the core community in cultivated turfgrass is likely distinct from that observed in other systems.

Delineation of a core microbiome is a crucial first step in assessing the overall health of a turfgrass ecosystem. Microbiota within the core microbiome can be used as biomarkers to assess how microbial communities respond to environmental stimuli, with the end goal of manipulating these communities to achieve a desired benefit (Shade and Handelsman 2012). For example, Verrucomicrobia bacteria have been identified as core constituents in native grassland ecosystems that decrease as a result of fertility inputs (Fierer et al. 2013). Furthermore, presence of Verrucomicrobia species has been positively correlated with genes involved in carbon cycling, and negatively associated with those involved in nitrogen cycling (Fierer et al. 2013). Determining whether Verrucomicrobia are present in turfgrass putting greens, and if they are impacted by fertility inputs, could give important insight into how nutrient cycling might be affected in this ecosystem. Alternatively, this data could be used to select for a microbial community similar to that observed in native grasslands to determine if this community is in anyway beneficial to the health of the cultivated turfgrasses.

Seasonality is known to affect microbial population counts and enzymatic function in bacterial and fungal populations, as well as decreasing metabolic activities, such as dehydrogenase, in perennial grassland ecosystems and agricultural soils during winter months (Cho et al. 2008, Dunfield and Germida 2003, Guicharnard et al. 2010). However, little is known about the impact of seasonality on microbial populations in cultivated turfgrass. In a bermudagrass (*Cynodon dactylon* L.) putting green, the population of one rhizosphere bacterium (*Stenotrophomonas maltophila)* was significantly different over a two year period (Elliot and Des Jardin 1999). Similarly, Yao et al. observed seasonal fluxes in microbial respiration and enzymatic activities in

six cultivated cool- [tall fescue (*Festuca arundinacea*), Kentucky bluegrass (*Poa pratensis*), creeping bentgrass] and warm-season [centipedegrass (*Eremochloa ophiuroides*), zoysiagrass (*Zoysia japonica*), bermudagrass] turfgrass species in North Carolina (2011). Thus, assessing the impact of microbial communities in putting greens over time requires further study.

Golf course putting greens, with their input-intensive management practices and perennial nature, represent a unique environment unlike any system observed in traditional agriculture. Understanding the microorganisms that are routinely found in the soil of turfgrass putting greens across seasons is the first essential step to gaining insight into how/if management practices could potentially be utilized to select for desirable microbial communities. Employing advanced molecular technologies, such as next-generation metabarcode sequencing, is an ideal way to rapidly identify the entire cohort of resident microorganisms in putting green turf. To this end, the objectives of this study were to 1) identify the complete collective of archaea, bacteria, and fungi inhabiting the soil of annual bluegrass putting green turf over 12 months, and 2) determine the constituents comprising the core microbiome found in all samples throughout this period.

MATERIALS AND METHODS

Experimental plots

A one-year field trial was initiated in 2014 on annual bluegrass (ABG) turf maintained as a putting green at the Rutgers Horticultural Research Farm No. 2 in North Brunswick, NJ, where the native soil profile is a Nixon sandy loam (Taxonomy = fine-loamy, mixed, mesic Type Hapludaults) with a pH of 5.9. Experimental plots received either (1) no N, (2) 4.9 kg N ha^{-1} every 7 d, (3), 4.9 kg N ha^{-1} every 14 d, (4) 4.9 kg N ha^{-1} every 28 d or (5) 9.8 kg ha^{-1} every 7 d. Each treatment was replicated four times. Nitrogen was applied as a foliar spray using urea. In 2014, N treatments began on 7 May and continued every 7, 14 or 28 d until 8 September. In 2015, treatments were applied on 5, 12, 19 and 26 May.

General Field Maintenance

Turf was mowed daily with a triplex greens mower (Models 3000-04350 and 3150-04357, Toro Co., Bloomington, MN) at a bench setting of 3.2 mm with clippings collected. Turf was irrigated with overhead irrigation and a hand-held syringe hose to maintain moderately dry conditions. Kiln-dried, medium-coarse, silica sand was applied as topdressing every 14 days at rates that matched the growth of the turf canopy. Four applications of urea ($CO(NH_2)_2$) at 4.9 kg N ha^{-1} were applied to all plots on 14 and 26 April, 22 September, and 6 October 2014 to promote turf growth and recovery from anthracnose disease. Additional macro- (calcium, magnesium, potassium, phosphorous) and micronutrients were applied as needed based on soil test results.

A disease management program to suppress summer patch (*Magnaporthiopsis poae*), brown patch (*Rhizoctonia solani*), dollar spot (*Sclerotinia homoeocarpa*) and

brown ring patch (*Waitea circinata*) was in place throughout the duration of the study. In 2014, dollar spot was preventatively controlled with vinclozolin [3-(3, 5-dichlorophenyl)-5-ethenyl-5-methyl-2, 4-oxazolidinedione] at 1.5 kg a.i. ha^{-1}, or boscalid {3-pyridinecarboxamide, 2-chloro-N-[4'-chloro(1,1'-biphenyl)-2-yl]} at 0.4 kg a.i. ha^{-1} every 14 d from 25 April to 23 August 2014. Fluoxastrobin {[(1E)-[2-[[6-(2-chlorophenoxy)-5-fluoro-4-pyrimidinyl]oxy]phenyl]-5,6-dihydro-1,4,2-dioxazin-3-yl) methanone-O-methyloximewere} at 0.44 kg a.i. ha^{-1} or flutolanil [N-(3-isopropoxyphenyl)-2-(trifluoromethyl)benzamide] at 6.4 kg a.i ha^{-1} was applied biweekly for brown ring patch control between 1 April and 6 May 2014. Flutolanil at 6.4 kg a.i. ha^{-1} was used every 14 d to suppress brown patch between 11 June and 9 August 2014. Summer patch was controlled with azoxystrobin [methyl (E)-2-{2-[6-(2-cyanophenoxy) pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate] at 3 kg a.i. ha^{-1} on 14 May and 11 June 2014. Chlorothalonil (tetrachloroisophthalonitrile) was applied at 12.6 3 kg a.i. ha^{-1} on 26 September and 6 October 2014 to control algae. In 2015, only boscalid at 0.4 kg a.i. ha^{-1} and fluoxastrobin 0.44 kg a.i. ha^{-1} were applied on 14 and 19 May 2015, respectively, to prevent spring diseases.

The growth regulators ethephon [(2-chloroethyl) phosphonic acid] and trinexapac-ethyl ethyl [4-(cyclopropyl-α-hydroxy-methylene)-3,5-dioxocyclohexanecarboxylic acid ethylester] were applied at 3.3 kg a.i. ha^{-1} and 0.05 kg a.i. ha^{-1}, respectively, on 4 and 21 April, and 6 May 2014 to suppress seedheads, followed by weekly applications of trinexapac-ethyl at 0.05 kg a.i. ha^{-1} from 14 May to 12 November 2014 to restrict vertical growth. In 2015, ethephon and trinexapac-ethyl were reapplied at the same rates on 2, 7,

and 27 May 2015, followed by a single application of trinexapac-ethyl at 0.05 kg a.i. ha^{-1} on 4 June 2015.

Chlorantraniliprole {3-bromo-N-[4-chloro-2-methyl-6-[(methylamino)carbonyl]phenyl]-1-(3-chloro-2-pyridinyl)-1H-pyrazole-5-carboxamide) was sprayed at 0.15 kg a.i. ha^{-1} on 14 May 2014 and bifenthrin {2-Methyl-3-phenylphenyl)methyl (1*S*,3*S*)-3-[(*Z*)-2-chloro-3,3,3-trifluoroprop-1-enyl]- 2,2-dimethylcyclopropane-1-carboxylate} was applied at 0.12 kg a.i. ha^{-1} on 25 May 2015 to control annual bluegrass weevils (*Listronotus maculicollis*).

Moss and crabgrass were controlled with the herbicides carfentrazone-ethyl {ethyl 2-chloro-3-[2-chloro-5-[4-(difluoromethyl)-3-methyl-5-oxo-1,2,4-triazol-1-yl]-4-fluorophenyl]propanoate} at 0.03 kg a.i ha^{-1} and fluazifop-P-butyl {butyl (R)-2-[4-[[5-(trifluoromethyl)-2-pyridinyl]oxy]phenoxy]propanoate} at 0.21 kg a.i. ha^{-1} on 14, 20, 27 and 30 October and 12 November 2014. All products (fungicides, growth regulators, insecticides, herbicides) were applied uniformly across all plots in this study.

Soil Sampling

Sampling sites measured 1.8 m by 1.8 m. Soil samples were taken three days after experimental N treatments were applied, except for the 16 April 2015 sampling date, which had no fertility treatments applied since 8 September 2014. No fungicides were sprayed at least 11 days prior to sampling. Soil was sampled by taking four 12.7 mm diameter by 50.8 mm depth soil cores (the approximate depth of annual bluegrass roots during much of the growing season) from four replicated plots for each of the five levels of N using a custom designed soil probe. Sampling occurred on 11 June, 25 July, and 27 August 2014 and 16 April and 3 June 2015. Plots were sampled within a 30 cm by

45 cm region located in the center of the plots. Soil cores were stored on ice immediately following removal, and screened through a 2.5 mm sieve before DNA extractions. Sieving removed all plant debris and large particulates, leaving the surrounding bulk soil for analysis.

Average monthly temperatures for each sampling date are available in Supplemental Table 1. Soil test results from July 2014, August 2014, and June 2015 are presented in Supplemental Table 2.

DNA Manipulations

The PowerSoil DNA Isolation Kit (Mo-Bio, Carlsbad, CA) was used for all genomic DNA extractions. Twelve blank DNA extractions (no sample included) were performed to serve as controls throughout the extraction and PCR processing. The PowerSoil protocol was modified to improve DNA yield and quality because the standard manufacturer's protocol consistently resulted in poor DNA yields (< 10 ng/µl), possibly due to the high sand content and minimal organic matter of our samples. In the PowerSoil Bead tube, 200 µl of bead solution was replaced with an equal volume of phenol:chloroform:isoamyl alcohol pH 8 (Fisher Scientific, Pittsburgh, PA), then 0.5 g of soil and 60 µl of Solution C1 were added. Tubes were shaken in a BioSpec bead-beater (BioSpec Products, Bartlesville, OK) on the medium setting for 1 min. The remaining steps of the PowerSoil protocol were followed according to manufacturer's recommendation for low biomass soil.

Amplicon libraries for Illumina sequencing were generated using a two-step PCR process: (1) amplification of the region-of-interest using targeted PCR primers that included overhang adapter sequences, and (2) the addition of unique barcode indices and

Illumina P5 and P7 sequence adapters. Organism-specific gDNA ribosomal markers were PCR amplified from (I) fungi, using the ITS1-F_KYO2 / ITS2_KYO2 and ITS3_KYO2 / ITS4 primer pair for the rDNA internal transcribed spacer region 1 and 2 (~300-bp; White et al. 1990, Toju et al. 2012), (II) bacteria, using the Ba9F/Ba515Rmod1 primer pair for the 16s rDNA (~500-bp; Weisburg et al. 1991, Kittelmann et al. 2013); and (III) archaea using the Ar915aF/Ar1386R primer pair for the 16s rDNA (~500-bp; Skillman et al. 2004, Watanabe et al. 2004). Unique overhang adapter sequences were added to the 5' end of each primer set to allow attachment of indices and Illumina sequencing adapters: forward primer= TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG; reverse primer= GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG. Reverse primers were synthesized in four different versions, with the addition of 0-3 mixed sequence bases (where N is any nucleotide) between the overhang adapter and the locus specific sequence to introduce sequence complexity. A complete list of PCR primers used in this study is available in Table 1. PCR reactions were performed using MangoTaq DNA Polymerase (BioLine, Taunton, MA) in 25 µl volumes containing 5x PCR buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP and 10 nM of each primer. Equal volumes of all four reverse primers were used to create a 10 nM working stock. The cycling conditions were as follows: 94°C for 2 min, followed by 30 cycles of 94°C for 1 min, 52°C for 45 s, 72°C for 45 s, followed by a final extension at 72°C for 5 min. Following PCR, amplicons were visualized on a 0.8% agarose gel and the four amplicons from each sample were pooled. Pooled amplicons were purified using the DNA (PCR) Clean and Concentrator Kit (Zymo Research, Irvine, CA).

Sequencing libraries were prepared using MangoTaq DNA Polymerase (BioLine, Taunton, MA) in 40 µl volumes containing 5x PCR buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP and 5 µl of each Nextera index primer (Illumina, San Diego, CA) to allow sample multiplexing. The cycling conditions were as follows: 72°C for 3 min, 95°C for 30s, followed by 12 cycles of 95°C for 10 s, 55°C for 30 s, 72°C for 30 s, followed by a final extension at 72°C for 5 min. Libraries were purified using the Zymo DNA (PCR) Clean and Concentrator Kit and quantified using the QIAxcel System (QIAGEN, Gaithersburg, MD) and Qubit fluorometer (Life Technologies, Grand Island, NY). Purified libraries were normalized to 4 nM and pooled into a single sample. To increase library complexity, a phiX control (Illumina) and an indexed gDNA library of the fungus *Colletotrichum graminicola* was spiked into the same run. Pooled libraries were denatured, diluted to 18 pM and *sequenced on Illumina's MiSeq platform using a 600 cycle MiSeq v3 Reagent Kit*. Paired end fastq files were generated and stored on Illumina's BaseSpace platform (https://basespace.illumina.com). To generate further data, two additional sequencing runs were performed on the MiSeq platform with 600 cycle chemistry, except pooled libraries were diluted to 12 pM. Sequencing data from all runs was combined and all downstream analyses were performed on the complete dataset.

Bioinformatic Analyses

The QIIME pipeline (version 1.9.1; Caporaso et al. 2010) was used for initial filtering and data analyses. All steps were performed on an Amazon EC2 image (Amazon machine image number: ami-1918ff72). Forward and reverse reads were stitched together using fastq-join in the ea-utils package (Aronesty 2011). Reads were only assembled if there were no base differences in the overlap region. Following

assembly, barcodes were removed and pooled reads were separated into each sample with the split_libraries_fastq.py script. Only sequences with a quality score > 20 and no ambiguous bases were retained. Following demultiplexing, LabVIEW (National Instruments, Austin, TX) was used to separate 16s and ITS reads. Sequences were checked for chimeras using the 64-bit version of USEARCH (Edgar et al. 2011). Any sequences identified as chimeric were removed from the dataset. A 97% sequence similarity threshold was used to cluster sequences into operational taxonomic units (OTUs) with a *de novo* picking strategy. Following OTU picking, any OTU representing a singleton was removed. Archaea and bacteria OTUs were assigned to a taxonomic rank with the RDP Classifier 2.2 (Wang et al. 2007) and the Greengenes database version 13_8 (McDonald et al., 2012, Werner et al. 2012). Fungal taxonomic rank was determined using BLAST (Altschul et al. 1990) with the BLASTn option and the UNITE + INSDC reference database version 7 (Abarenkov et al. 2010). In order to determine the distribution of common microbial pathogens of annual bluegrass that were not represented by the UNITE + INSDC database, a custom database composed of ITS sequence data from 41 fungal and oomycete pathogens of turfgrass was compiled from published sequence data available from NCBI GenBank and in-house sequence data generated for previous studies (submission of new data to GenBank is in progress). The database was inserted into QIIME and pathogen identification was determined using the RDP Classifier 2.2 (Wang et al. 2007).

Data parsing scripts developed in C++ were used to remove any OTUs that could not be identified using the Greengenes or UNITE +INSDC databases. Taxonomic assignments discussed in this chapter represent the lowest rank that could be determined

for a given OTU. Some bacteria are described as candidate divisions, representing lineages of organisms with no cultured representative, but are distinct from other known lineages based on DNA sequence data (Hugenholtz et al. 1998).

Data Analyses

Detrended correspondence analysis was conducted using the Vegan package in R. Rarified datasets were used for all analyses, where the multiple_rarefactions.py script was used to rarify 16s sequences to a depth of 4500 (100 step increments, 10 replicates) and ITS sequences to a depth of 3000 (100 step increments, 10 replicates). The Shannon index was used to assess biodiversity across all samples. Nonparametric two-way t-tests utilizing Monte Carlo permutations to calculate the p-value were performed to compare alpha diversity calculations. A p-value less than 0.05 was considered significant. Diversity between samples (beta diversity) was calculated using Bray-Curtis dissimilarity matrices. Large values indicate that samples are not similar to one another in species composition (Gardener 2014). Cluster based neighbor-joining trees were generated from Bray-Curtis matrices. Analysis of similarities (ANOSIM) was used to test if samples within categories in dissimilarity matrices were more similar to one another than samples in different categories using 999 permutations.

Rank abundance plots were produced using the plot_rank_abundance_graph.py script for individual samples and fertility treatments. Rank abundance curves provide a way to visualize species abundance between samples. Species are ordered from most to least abundant on the x-axis, and the abundance of each type is plotted on the y-axis (Hughes et al. 2001). A steep slope indicates a community where species are not very

evenly distributed, as high-ranking species (left on x-axis) have much higher abundance (Hughes et al. 2001).

The core microbiome was identified using the compute_core_microbiome.py script, where core OTUs were defined as OTUs that are present in 100% of samples. For all analyses, 16s and ITS data was analyzed separately.

Data Availability

All sequence data is available under accession number SRP063317 in NCBI's Sequence Read Archive.

Supplemental Tables are available from www.eden.rutgers.edu/~lbeirn/dissertation.

RESULTS

Sequence Data

Three runs of Illumina sequencing of the PCR amplicons across the 25 samples (five pooled treatments collected at five time points) generated 3.84 X 10^7 total reads (run 1=1.06 x 10^7 reads, run 2=8.92 x 10^6 reads, run 3=1.00 x 10^7 reads). A total of 2.00 X 10^7 reads passed quality filtering (run 1=1.00 x 10^7 reads, run 2=8.53 x 10^6 reads, run 3=9.52 x 10^6 reads). After read stitching and demultiplexing, an average of 1.31 x 10^5 sequences per sample were generated from the combined three runs. From the complete set of reads, 18.9% of the total sequences were identified as chimeras and removed from the dataset, leaving 3.12 X 10^7reads for community analyses.

Kitome Analysis

Sequencing of blank samples from the PowerSoil DNA extraction kit generated a kitome (kit microbiome) that included 34 unique bacterial taxa and two unique fungal taxa. Removal of singletons from the kitome eliminated all fungal taxa and 11 bacterial taxa. The remaining 23 bacterial taxa represented five phyla: Acidobacteria (three taxa), Actinobacteria (four taxa), Chloroflexi (one taxon), Firmicutes (four taxa), and Proteobacteria (11 taxa). Kitome percent abundance averaged across all samples ranged from 1.31 X 10^{-3} (most abundant; *Pseudomonas sp.*) to 1.26 X 10^{-5} (least abundant; *Enhydrobacter sp.*). Remaining kitome taxa were removed from all downstream analyses by quality filtering. A complete list of taxa present in the kitome is listed in Table 2.

Microbial Community Analysis

In total, 1.50 X 10^5 OTUs were identified from all samples, of which 1.03 X 10^5 were archaea/bacteria and 4.66 X 10^4 were fungi. Archaea, bacteria and fungi were

identified from all samples. On average, 0.35% of the sequences were archaea, 10.61% of the sequences were bacteria and 0.10% of the sequences were fungal. The remaining 2.65×10^{-5} and 3.09×10^{-6} OTUs represented plant and protist DNA, respectively.

Diversity

Alpha diversity calculated using the Shannon index is summarized in Table 3. Nonparametric two-way t-tests showed no significant difference in alpha diversity between the five fertility treatments ($p = 0.6\text{-}1.0$) for either archaea/bacteria or fungi. When diversity values for N treatments were averaged and analyzed by sampling date, two pairwise comparisons were significant. Samples collected on 3 June 2015 exhibited significantly higher archaea/bacteria community diversity compared to the 11 June 2014 ($p = 0.03$) and 16 April 2015 ($p = 0.01$) sampling dates (Shannon index 10.93 versus 10.46 and 10.60, respectively). No sampling dates were significantly differentiated for the fungal community (Shannon index = 7.34- 8.39; $p = 0.15\text{-}1.0$). Overall, archaea/bacteria diversity was significantly higher than fungal diversity ($p = 0.006$).

Rarefaction curves plateaued for all samples, indicating that species diversity was adequately captured with our sampling methods (Figure 1A archaea/bacteria; Figure 1B fungi). Rank abundance plots revealed a similar slope for all samples collected on each of the five sampling dates for archaea/bacteria and fungi (Figures 2A-E). This indicates that within each respective fertility treatment, microbial populations from different sampling months display similar richness (number of taxa) and evenness (proportions of taxa).

In only a few cases were differences in the abundance individuals observed between sampling months. For example, in the 4.9 kg ha^{-1} N treatment applied every 28

days, the most abundant archaea/bacteria species (furthest left on the x-axis) from the 3 June 2015 sample, displayed fewer individuals compared to other sampling months (Figure 2B). Likewise, the 11 June 2014 and 16 April 2015 sampling dates in the 4.9 kg ha^{-1} N treatment applied every 7 days contained a higher number of their most abundant species compared to the other sampling dates (Figure 2D). For fungi, only the 16 April 2015 sample from the 4.9 kg ha^{-1} N treatment applied every 14 days, displayed higher counts of the most abundant species (Figure 3C).

For archaea/bacteria, all samples shared some species in common, as demonstrated by Bray-Curtis (BC) dissimilarity matrix values ranging between 0.64 and 0.89 (Table 4). If all species were shared between samples, BC dissimilarity values would equal zero. However, in general, samples were most similar in species composition to those collected in the same month, with samples collected from the same month all displaying low BC values (Figure 4). All samples from 25 July and 27 August 2014 sampling dates grouped within the same clade and clustered with their respective sampling month, except the untreated plots from 27 August 2014 and the high rate of nitrogen from 11 June 2014, which formed a subgroup within this clade (Figure 4). Likewise, all 11 June 2014, 16 April 2015, and 3 June 2015 samples clustered together, except for the 9.8 kg ha^{-1} N treatment applied every 7 days from 11 June 2014, the untreated 3 June 2015 sample, 4.9 kg ha^{-1} N treatment applied every 14 days from 3 June 2015, and 4.9 kg ha^{-1} N treatment applied every 28 days from 16 April 2015, which all formed their own clade closest to the remaining 3 June 2015 and 16 April 2015 samples. When analyzed by sampling month, the null hypothesis, that all microbial populations are the same, was rejected ($p = 0.001$, test statistic = 0.600).

Like archaea/bacteria, all fungal samples shared some species in common across samples (BC dissimilarity = 0.39 to 0.83) (Table 5), but once again samples were most similar in overall community composition to those collected in the same month (Figure 5). Samples from 11 June 2014, 25 July 2014, 27 August 2014, and 16 April 2015, all formed distinct clades. As with archaea/bacteria, samples collected during the hot summer months (25 July 2014, and 27 August 2014) grouped together. All samples collected in June 2014 and June 2015 formed their own individual clades and grouped together, except the sample from the 9.8 kg ha^{-1} N treatment applied every 7 days on 11 June 2014, the 3 June 2015 untreated check, and the 4.9 kg ha^{-1} N treatment applied every 14 days sample from 3 June 2015, which formed their own clade at the bottom of the tree and grouping with the 16 April 2015 samples (Figure 5). The null hypothesis was also rejected for seasonal categories ($p = 0.001$, test statistic = 0.781).

Detrended correspondence analysis (DCA) showed the archaea/bacteria samples loosely clustering by collection month (Figure 6); however, samples from 11 June 2014 were more removed from all other samples regardless of treatment, while samples from 25 July 2014, 27 August 2014, 16 April 2015, and 3 June 2015 grouped more closely together (Figure 6). No obvious clustering patterns were discernible for fungi (Figure 7).

Archaea/Bacteria Community Composition

In total, four archaeal and 33 bacterial phyla were identified. Within just the archaeal sample, total abundance averaged across all samples was as follows: Crenarchaeota 0.58%, Euryarchaeota 0.07%, an unidentified archaeal phyla 0.04% and Parvarchaeota 0.01% (Supplemental Table 1).

In total, 17 unique archaeal taxa were identified across all samples, which represented five classes in the Crenarchaeota, two classes in the Euryarchaeota, one class in the Parvarchaeota and one unidentified archaeal class. The Crenarchaeota comprised 53% of all archaea identified, followed by Euryarchaeota (29%), Parvarchaeota (12%) and an unidentified archaeal clone (6%). In the Crenarchaota, the Thaumarchaeota was the most dominate class, with two orders (Cenarchaeales, Nitrososphaerales) representing three unique families identified and four genera. In the Euryarchaeota, the class Methanobacteria was most frequent, representing one family and four genera. In the Parvarchaeota, only class Parvarchaea was present, representing two candidate family divisions. Averaged across all samples, all four archaea phyla were found in each sampling month (Table 6). For archaea, Crenarchaeota were most abundant in 11 June 2014 and 3 June 2015, with abundance totaling 7.77×10^{-3} and 7.64×10^{-3} in 3 June 2015, respectively, and lowest in July 2014 (abundance = 3.68×10^{-3}). Similarly, Parvarchaeota was most abundant in 11 June 2014 (3.12×10^{-4}), and lowest in 25 July 2014 (6.14×10^{-5}). Contrastingly, Euryarchaeota was most abundant in August 2014 (1.40×10^{-3}) and lowest in 11 June 2014 (1.48×10^{-4}).

Only one archaeal OTU, OTU536, representing an uncultured clone of Cenarchaeales SAGMA-X, was shared across all 25 samples (Table 7).

Within the bacterial sample, total abundance averaged across all samples was as follows: Proteobacteria 8.1% and Acidobacteria 7.8%. The remaining 31 bacterial phyla were present in abundance <1%. A complete list of bacterial phyla identified is available in Table 8. Across all samples, bacterial taxonomy could be summarized to 442 unique bacterial taxa (Supplemental Table 1). The phyla Proteobacteria dominated the unique

organisms identified, comprising 160 different taxa (36.2% of all bacteria identified). This was followed by, in order of decreasing representation: Actinobacteria (50 taxa; 11.3% of total bacteria), Acidobacteria (37 taxa; 8.3% of total), Chloroflexi (34 taxa; 7.6% of total), Bacteroidetes (23 taxa; 5.2% of total), Verrucomicrobia (21 taxa; 4.8% of total), Cyanobacteria (18 taxa; 4.1% of total), Planctomycetes (17 taxa; 3.8% of total), Firmicutes (12 taxa; 2.7% of total), candidate division OP11 (10 taxa; 2.3% of total), Armatimonadetes (9 taxa; 2% of total), Gemmatimonadetes (8 taxa; 1.8% of total), candidate division TM7 (8 taxa; 1.8% of total), Chlorobi (5 taxa; 1.1% of total), candidate division OD1 (4 taxa; 0.9% of total), Elusimicrobia (3 taxa; 0.7% of total), Nitrospirae (3 taxa; 0.7% of total), candidate division GNO2 (2 taxa; 0.5% of total), candidate division OP3 (2 taxa; 0.5% of total), Spirochaetes (2 taxa; 0.5% of total), and candidate division TM6 (2 taxa; 0.5% of total). The remaining phyla, Fibrobacteres, Tenericutes, and Thermi, and candidate divisions BHI80-139, FBP, FCPU426, Kazan-3B-28, MVP-21, SBR1093, SR1, TPD-58, and WPS-2, only represented one unique taxonomic group out of the 442 total identified (0.2% of total).

The phyla with the largest number (count and abundance) of OTUs identified, the Proteobacteria, could be further broken down into Alphaproteobacteria (83 taxa), Betaproteobacteria (32 taxa), Gammaproteobacteria (25 taxa), Deltaproteobacteria (18 taxa) and candidate division TA18 (2 taxa) (Supplemental Table 1). Alphaprotebacteria was represented by ten taxonomic orders of bacteria, of which Rhizobiales dominated (38 taxa). Betaproteobacteria was composed of eight taxonomic orders, with Burkholderiales recovered most frequently (16 taxa). Gammaproteobacteria contained nine taxonomic

orders, with the order Legionellales representing eight of those taxa. Deltaproteobacteria contained eight taxonomic orders, with Myxococcales dominating (nine taxa).

The phylum Actinobacteria, though representing 50 taxa, was only represented by five taxonomic classes (Acidimicrobiia, Actinobacteria, candidate division OPB41, Thermoleophilia, and an unidentified class), and seven orders (Acidimicrobiales, Actinomycetales, Gaiellales, Solirubrobacterales, and three unidentified orders), with Actinomycetales dominating (36 taxa). Acidobactera was represented by of 13 taxonomic classes, representing 22 taxonomic orders. The order Acidobacteriales dominated this group, representing seven taxa, followed by Solibacterales, with six taxa. In total, 114 bacterial genera were identified, not including candidate divisions (Table 9).

Twenty-seven bacteria phyla were present in all sampling months (Table 6). Only candidate divisions OP11 and SR1 were most abundant in 11 June 2014 (1.13×10^{-2} and 2.39×10^{-3}, respectively), while ten phyla peaked in abundance in 25 July 2014 (Chlorobi, Elusimicrobia, Nitrospirae, Planctomycetes, Proteobacteria, Spirochaetes, Verrucomicrobia and candidate divisions GN02, and TM6). In 27 August 2014, four phyla peaked in abundance - Fibrobacteres, Gemmatimonadetes and candidate divisions FCPU426 and OD1. In 16 April 2015, Cyanobacteria, Firmicutes, and candidate divisions MVP-21 and TM7 were most abundant, while Acidobacteria, Actinobacteria, Armatimonadetes, Bacteroidetes, Tenericutes, and candidate divisions FBP and WPS-2 peaked in abundance in 3 June 2015. Six bacterial taxa (Thermi and candidate divisions BHI80-139, FCPU426, Kazan-3B-28, OP3 and SBR1093) were not found in every sampling date. Thermi was found in only 16 April and 3 June of 2015. In addition, Kazan-3B-28 was found only in 11 June and 25 July 2014, OP-3 was present in just 2014

samples, SBR1093 was only in 25 July 2014 and 16 April 2015, and BHI80-139 only in 27 August 2014 samples.

For bacteria, 30 bacterial OTUs were shared across all 25 samples (Table 7).

Fungal Community Composition

Seven fungal phyla (Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota, Rozellomycota, Zygomycota, and one unidentified fungal phylum) and one protist phylum (Cercozoa) were identified in this study. Total abundance averaged across all samples, was less than 1% for all phyla. Members of the Ascomycota were present in the highest abundance, at 0.19%, followed by Rozellomycota (0.01%), Chytridiomycota (0.002%), an unidentified fungal phylum (0.0003%), Zygomycota (0.00021%), Basidiomycota (0.0020%), and Glomeromycota (0.00017%) (Supplemental Table 2).

In the Ascomycota, two fungal classes were identified: Dothideomycetes and Sordariomycetes (Supplemental Table 2). This represented three orders: Myriangiales, Bolinales, and an unidentified order in the Soradriomycetes. In the Basidiomycota, the lowest taxonomic level identified was one Agaricomycetes class, representing an order in the Agaricales. For Chytridiomycota, three fungal classes were present, but only one, Blastocladiomycetes, could be classified taxonomically. At the genus level, this represented *Blastocladiella spp*. In the Glomeromycota, only the order Paraglomerales in the Glomeromycetes was discovered. For Zygomycota, only *Mortierella sp.* was identified. No lower taxonomic assignments were possible for the Rozellomycota.

Only three fungal groups were found in each month: Rozellomycota, Chytridiomycota, and Myriangiales (Table 10). Averaged across all sample,

Myriangiales was most abundant in the two June sampling dates (11 June 2014 = 1.92 X 10^{-3}; 3 June 2015 = 5.22 X 10^{-3}) and least abundant in the three remaining sampling dates (25 July 2014 = 9.34 X 10^{-4}; 27 August 2014 = 7.91X10^{-4}; 16 April 2015 = 5.46 X 10^{-4}). The unidentified Chytridiomycota was most abundant in 16 April 2015 (2.08 X 10^{-5}), followed by 25 July 2014 (1.47 X 10^{-5}), 11 June 2015 (1.29 X 10^{-5}), 27 August 2014 (1.02 X 10^{-5}), and 11 June 2014 (2.14 X 10^{-6}). Rozellomycota abundance was as follows- 27 August 2014 = 1.85 X 10^{-4}, 25 July 2014 = 1.66 X 10^{-4}, 11 June 2014 = 1.37 X 10^{-4}, 16 April 2015 = 1. 28 X 10^{-4}, and 3 June 2015 3.04 X 10^{-5}.

Four fungal groups were found only in certain months: unidentified Sordariomycete (11 June 2014 = 5.3X 10^{-6}), *Blastocladiella sp*. (3 June 2015 = 1.14 X 10^{-5}), unidentified Chytridiomycota (16 April 2015 = 6.75 X 10^{-6}), and *Mortierella sp.* (16 April 2015 = 1.08 X 10^{-5}). The remaining four fungal groups were present in three out of five sampling dates (Boliniales, unidentified fungal phylum) or two out of five sampling dates (Agaricales, Paraglomerales).

Only one fungal OTU, OTU269, representing a fungal species in the Myriangiales, comprised the core microbiome in all 25 samples collected (Table 7). However, the overall abundance of OTU269 was fairly low, with abundance less than 0.5% across all samples.

Turfgrass Pathogen Distribution

Five foliar turfgrass pathogens were identified in soil samples to the genus level using the custom designed turfgrass pathogen database (available from www.eden.rutgers.edu/~lbeirn/dissertation/chapter2). The root-infecting turf pathogens *Magnaporthiopsis poae* and *Gaeumannomyces graminis* or other soil-borne pathogens

commonly associated with putting greens in the northeastern U.S.A. were not identified using the custom turf database. However, *Magnaporthe* and *Gaeumannomyces spp.* were identified at low levels in the UNITE + INSDC database ($<10^{-6}$). Relative to all other turfgrass pathogens, the fungus *Microdochium nivale*, the causal agent of pink snow mold, and the fungus *Sclerotinia homoeocarpa*, the causal agent of dollar spot disease, were present in the highest abundance in all samples, at levels of 1.44×10^{-4} and 8.29×10^{-5}, respectively. *Colletotrichum cereale*, the incitant of the foliar and stem rot disease anthracnose, *Puccinia sp.*, the causal agents of rust diseases, and *R. solani*, causal agent of brown patch disease were present, but at low levels (2.72×10^{-5}, 3.44×10^{-6}, and 2.28×10^{-6}, respectively).

DISCUSSION

The primary objective of this study was to identify the resident microbial community in the soil of annual bluegrass putting green turf throughout the growing season when exposed to different rates of nitrogen fertilizer. Across all five N rates, collected at five different time points, a diverse, species-rich microbial community comprised of archaea, bacteria and fungi in just 12.5 g of soil was uncovered. In fact, the large number of OTUs identified here is equal to or greater than that described from many agricultural or natural ecosystems. For example, 1.03×10^5 archaea/bacterial OTUs were identified, while approximately 3.3×10^4 and 5.3×10^4 OTUs have been reported for sugar beet and potato agroecosystems, respectively (Inceoglu *et al.* 2011, Mendes et al. 2011). In the Amazonian forest, Mojave Desert and the Konza Praire, 1.0×10^4 archaea/bacterial OTUs have been reported from 10 g of soil (Fierer et al. 2007). Similarly, 44 phyla in total were identified, comparable with the 48 reported from poplar (*Populus deltoides*) tree stands (Shakya et al. 2013). In addition, the dominant phyla observed in this study (Crenarchaeota, Proteobacteria, Acidobacteria, Ascomycota), are also the dominant phyla reported in many other soil ecosystems, such as agricultural fields and native grasslands (Dunfield et al. 2003, Cho et al. 2008, Guicharnaud et al. 2010, Fierer et al. 2012), indicating that manmade ecosystems can share similarities with natural sites. Of course, since different methodologies were used to identify the microbial communities in these studies, caution should be exercised when making comparisons. Likewise, it is not yet known if microorganisms shared between natural systems and highly managed anthropogenic landscapes are functioning in the same manner, or how these communities might vary at the species level. Nevertheless, it is

important to note that soil of annual bluegrass putting green turf supports thousands of microorganisms, and is not as an inhospitable environment as is often assumed (Elliot et al. 2008).

Fourteen bacterial genera identified here have also been reported from the rhizosphere of creeping bentgrass and bermudagrass putting greens in the southeastern United States (Elliot et al. 2008). Three of these genera, *Brevibacterium, Burkholderia,* and *Pseudomonas,* possess interesting metabolic capabilities. For example, members of the Burkholderiales and Actinomycetales represent bacterial groups known to produce antibiotics (Pidot et al. 2014, Lazzarini et al. 2000), thus they may possess compounds of interest for fighting plant diseases. Likewise, *Pseudomonas* species have been successfully used as biocontrol agents in cotton (*Gossypium hirsutum*), tobacco (*Nicotiana tabacum*), radish (*Raphanus sativus*), and potato (*Solanum tuberosum*) (reviewed by Weller 2007) or plant-growth promotors in rice (*Oryza sativa*) (Noori and Saud 2012). In fact, bacteria in the Pseudomonadaceae and Burkholderaceae have been correlated with disease-suppressive soils in sugar beet and other agricultural systems, where there presence has been shown to suppress *R. solani* (Mendes et al. 2011). A similar effect has been seen in wheat, where Pseudomonads have been used to suppress take-all disease, caused by the fungus *Gaeumannomyces graminis* var. *tritici* (Weller et al. 1988; reviewed in Weller et al. 2002). Both *G. graminis* and *R. solani* are also pathogens of turfgrass, causing the diseases such as take-all and brown patch, respectively (Smiley et al. 2005). Interestingly, only very low levels of foliar turf pathogens (*Colletotrichum cereale, Puccinia sp., R. solani*) were detected, as might be expected from bulk soil samples, but root infecting pathogens such as *Magnaporthiopsis*

or *Gaeumannomyces* were not identified at all. The low levels of turf pathogens seen in this study could be attributed to extensive fungicide use at this site over the past decade, or they may have been removed with roots and other plant debris during the soil sieving process. However, the identification of organisms capable of behaving as antagonists (potential biocontrol agents) in our study is promising, and investigations targeting these specific organisms should be pursued. The recovery of these microorganisms from putting greens in both the northeastern and southeastern United States suggests that they can tolerate a wide-range of habitats, soil conditions, temperatures and management practices including extensive pesticide use. As such, they may hold promise as potentially beneficial microorganisms that could be utilized in a variety of environments, including turfgrass or agricultural systems.

Several other interesting taxa were identified in our study. In particular, the frequency and widespread presence of the Crenarchaeota and Paraglomerales suggests an important role for these microbes in the turfgrass system. Paraglomerales represents a newly described group of arbuscular mycorrhizae (Oehl et al. 2011) and are therefore another microorganism that could potentially contribute to plant health and ecosystem function. Crenarchaeota are the most abundant phyla of ammonia-oxidizing microorganisms in terrestrial habitats, where they can reduce ammonia to nitrite for use by the plant (Leininger et al. 2006). For plants to uptake urea (the N source used in my study), it must first be broken down into ammonia. From here, ammonia must react with water to form ammonium in the soil, be lost to the atmosphere, or be converted to nitrite. Recently, many ammonia-oxidizing archaea have been shown to possess urease genes, possibly as a way to facilitate nitrification (Alonso-Saez et al. 2012). In addition, gene

expression studies have also shown increased expression of *amoA*, the archaeal gene involved in the first step of ammonia oxidation, in the presence of urea (Lu et al. 2012), suggesting these microbes are capable of utilizing urea to begin ammonia oxidation. Further investigations of how ammonia-oxidizing archaea affect the nitrogen cycle in turfgrass putting greens will be necessary to fully understand how such organisms might be used to improve N use efficiency in cultivated turfgrasses. Likewise, the presence of Verrucomicrobia could be indicative of an organism involved in carbon cycling. At present, the biology of Verrumicrobia is not well understood due to its slow growth in culture; however there has been a positive association with their abundance and carbohydrate metabolism genes in tallgrass prairie systems (Fierer et al. 2013). This suggests a role in degrading plant material, thus Verrucomicrobia may be important for breaking down dead plant tissue in the turfgrass system, however, further research will need to be conducted to verify this theory.

Of course, additional data is required to evaluate the function of the microbes identified in the current study and if in fact they do represent living organisms. Extracellular DNA from dead or decaying organisms has been shown to persist in the soil for up to two years, though this often reflects data from controlled greenhouse or laboratory experiments (Nielsen et al. 2007). Currently, we cannot confirm that the DNA extracted in our study represents that of living organisms. However, the samples collected here were exposed to a wide range of temperatures, chemicals, and naturally occurring nucleases and microorganisms that could all degrade free standing DNA quickly (Nielsen et al. 2007), thus it is likely that our sample may contain very little DNA from non-viable organisms. Moving forward, gene expression studies, combined with

techniques to target DNA from living organisms, such as those involving propidium monoazide to discriminate between viable and nonviable DNA (Weinmaier et al. 2015), should be employed to determine microbial function in envrionmental systems. Nevertheless, the organisms reported here serve as a starting point to conduct future research investigating the link between microbial function and plant health in this system.

From the entire microbial community, 32 OTUs were identified as belonging to the core microbiome in our study. These organisms were found in all fertility treatments and sampling dates and as such provides important baseline information about the microorganisms consistently found in the soil of annual bluegrass putting green turf. For example, as discussed above, the presence of SAGMA-X-like archaea in all samples suggests the possibility that it plays a major role in the nitrogen cycle of turf and may provide a way to examine nitrification and how the nitrogen cycle is impacted by input-intensive management regimes. Similarly, a bacterium representing candidate division OP11 was part of the core microbiome in this study. This bacterium is believed to have a role in carbon cycling in terrestrial habitats, due to the polymer-degrading enzymes it possesses (Youseff et al. 2011), and thus should be investigated further in the turfgrass rhizosphere.

In addition to identifying core OTUs that could be utilized as indicators of plant health within annual bluegrass putting green turf, bacteria that have been reported as core OTUs in other systems were also identified. The bacterial core microbiome in our study largely consisted of Proteobacteria and Acidobacteria, a result that has also been documented in Arabidopsis, sugarcane, poplar stands, and alpine bogs (Lundberg et al. 2012, Shakya et al. 2013, Bragina et al. 2015, Yeoh et al. 2015). Within the

Proteobacteria, five families − Bradyrhizobiaceae, Hyphomicrobiaceae, Rhodospirillaceae, Sinobacteraceae, and Xanthomonadaceae − are shared between the *Arabidopsis thaliana* core microbiome (Lundberg et al. 2012) and the soil of annual bluegrass putting green turf. For Acidobacteria, only Candidatus *Solibacter* was shared between our study and mature poplar stands (Shakya et al. 2013). The presence of shared organisms across plant hosts and ecosystems is interesting, and suggests a universal role for these bacteria in plant-microbe interactions in multiple ecosystems. In the future, these universally distributed microorganisms could be utilized to conduct comparisons between manmade and natural ecosystems, by investigating gene expression across sites.

Seasonality strongly influenced microbial populations in our study. For example, samples collected in the same month generally displayed similar species compositions compared to those collected in different times of the year. Microbial diversity was significantly different when examined by sampling month. The 3 June 2015 archaea/bacteria sample displayed higher diversity compared to samples from 11 June 2014 and 16 April 2015, a finding that is most likely attributable to the high abundance of Crenarchaeota and seven bacterial phyla that peaked in 3 June 2015. These phyla were present in all samples, thus it is likely that their varying abundance in specific sampling months, not their presence/absence, is contributing to the variations observed in alpha diversity in sampling dates. This is not surprising, as bacterial and fungal populations are known to vary in response to temperature in perennial ecosystems (Cho et al. 2008, Dunfield and Germida 2003), and changing temperatures are known to impact metabolic activities in microorganisms (Guicharnard et al. 2010). Interestingly, species identified from samples of archaea/bacteria and fungi collected during the hot summer months (25

July and 27 August 2014) in the current study typically grouped together, as did species from samples collected in the cooler months (11 June 2014, 3 June 2015, and 15 April 2015).

While environmental temperature may have contributed to the clustering of samples collected during the same sampling months, we cannot ignore the role the host plant may be playing. In natural settings, annual bluegrass maintains a bunch-type phenotype and plants produce a copious amount of seeds in early spring (Mao and Huff 2012). On golf courses, annual bluegrass plants exhibit a more perennial form (Huff 2013), and seed heads are often suppressed with the use of plant growth regulators. The suppression of flowering in annual bluegrass on golf course putting greens could divert resource allocation into other areas, such as plant growth and maintenance, altering overall plant phenology. As such, the microbial community in the rhizosphere of annual bluegrass putting green turf from April and June samples could be responding to the overall changes in plant phenology induced by suppressing flowering, thereby explaining their clustering in phylogenetic trees. In addition, annual bluegrass does not tolerate heat stress well, and when exposed to continual temperatures above 27°C, root function is reduced and plants can begin to wilt and yellow (Beard et al. 1978). Root exudates are known to influence microbial communities in the rhizosphere, with certain compounds selecting for specific groups of microorganisms (Bais et al. 2006). Thus, the similarities observed between microbial communities sampled from July and August in 2014 could be a result of reduced root exudate production by annual bluegrass. In addition, the high temperatures occurring during these months could be encouraging root death, favoring microorganisms that can degrade dying plant material.

While seasonality played an important role in influencing microbial communities, we did observe a few exceptions among our sample collection. Three samples did not cluster with their respective sampling month for both archaea/bacteria and fungi (9.8 kg ha^{-1} N every 7 days from 11 June 2014, untreated check from 3 June 2015, 4.9 kg ha^{-1} N every 14 days from 3 June 2015). Upon closer examination, six bacterial phyla (candidate divisions BH180-139, Kazan-3B-28, MVP1, OP3, SBR1093, TPD-58) and four fungal phyla (Basidomycota, Glomeromycota, Zygomycota, unidentified fungal phylum) were absent entirely from these samples, explaining their clustering in neighbor-joining trees with one another. The absence of these phyla is unlikely the result of fertility treatments, as all three samples experienced no or very different nitrogen applications. Furthermore, these phyla are present in other samples from 11 June 2014 and 3 June 2015, thus their absence here is suspect. Currently, we are not able to determine if these phyla were simply missed upon sampling, or if their absence could be a result of other environmental factors, such as slight deviations in field conditions. Despite the deviations of these three samples, the overwhelming influence of seasonality is apparent in the remaining samples.

In addition to nitrogen fertility inputs, the study site also received regular fungicide applications to control common turfgrass diseases, in a manner that would be employed on professional golf course greens. Although fungicide applications have been shown to reduce populations of nonpathogenic fungi (Smith et al. 2000, Sigler and Turco 2002) over time, we did not observe a consistent decrease in abundance or diversity of such fungal over a 12 month period that could correlate with fungicide use. Likewise, we did not see fungal populations increase in abundance when no fungicides were applied.

Only one OTU, identified as *Mortierella sp.*, was found in abundance in 16 April 2015, when no fungicide applications had been applied since the previous autumn. *Mortierella sp*. is a common soil inhabitant that is known to degrade herbicides (Ellegaard-Jensen et al. 2013), thus the presence of this fungus at just one time point in April 2015 could be linked to five late-season herbicide applications between 14 October and 12 November 2014, rather than fungicide usage.

In conclusion, while a core microbiome inhabiting the soil of annual bluegrass putting green turf in the Northeastern United States was identified, the resident microbial community identified here comes from one location over a one year period and thus may not reflect the core microbiome of other turfgrass systems. However, it represents a starting point for future research examining microbial communities in cultivated turfgrass. Plant hosts are known to strongly influence soil microbial communities, often through the secretion of species-specific root exudates (Broeckling et al. 2008, el Zahar Haichar et al. 2008); consequently, core microbial communities in turfgrass may vary with different turfgrass species. In addition, the strong affect of seasonality on microbial populations uncovered in our study may have less influence in regions where warmer climates promote a year-round growing season, or where temperature and other environmental parameters are less variable throughout the year. Culture-based analysis of bacterial populations in creeping bentgrass putting greens in Alabama and North Carolina and bermudagrass greens in Florida and South Carolina sampled over four years revealed significant effects of sampling date, turfgrass host, and site location (Elliot et al. 2004). Yet, sampling date represented less than 10% of the observed differences overall in their studies, with site location and host plant accounting for most of the variation

(Elliot et al. 2004). Thus, seasonality, although influential, is not the only factor affecting microbial diversity and species composition in turfgrass systems. Regardless, additional geographic sites and turfgrass hosts will need to be sampled before the core microbial community in all turfgrass stands can be well understood.

This study represents the first next-generation sequence based analysis of microbial communities in annual bluegrass putting green turf, highlighting a wide array of microorganisms spanning three Kingdoms. While the functional role of the microbiota identified here is not yet known, this study will serve as a starting point for investigating important questions about the ecology of this system, such as biogeochemical cycling, feedback mechanisms, and overall turfgrass sustainability. Most importantly, armed with this technology, researchers will now be able to target specific organisms of interest in the soil of annual bluegrass putting green turf and determine what role, if any, these communities may play in promoting healthy plants.

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Table 1. PCR primers used in this study.

Primers	Description	Sequence (5' to 3')	Reference
Ar915aF	Archaea 16s rDNA forward primer[a]	AGGAATTGGCGGGGGAGCAC	41
Ar1386R	Archaea 16s rDNA reverse primer[b]	GCGGTGTGTGCAAGGAGC	41
Ba9F	Bacterial 16s rDNA forward primer[a]	GAGTTTGATCMTGGCTCAG	40
Ba515Rmod1	Bacterial 16s rDNA reverse primer[b]	CCGCGGCKGCTGGCAC	40
ITS3_KYO2	Fungal ITS2 forward primer[a]	GATGAAGAACGYAGYRAA	38
ITS4	Fungal ITS2 reverse primer[b]	TCCTCCGCTTATTGATATGC	39
ITS1F_KYO2	Fungal ITS1 forward primer[a]	TAGAGGAAGTAAAAGTCGTAA	38
ITS2_KYO2	Fungal ITS1 reverse primer[b]	TTYRCTRCGTTCTTCATC	38

[a]Forward overhang adapter TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG was appended to the 5' end of all forward primers
[b]Reverse overhang adapter GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-(N, NN, NNN) was appended to the 5' end of all reverse primers. Reverse primers were synthesized in four different versions, with the addition of 0-3 mixed sequence bases (N) between the overhang adapter and the locus specific sequence to introduce sequence complexity.

Table 2. Operational taxonomic units (OTUs) identified from the MoBio PowerSoil DNA extraction kit.

	OTU ID[a]	Taxonomy	Present as Singleton[b]
Archaea			
	NA[c]	NA[c]	NA[c]
Bacteria			
	OTU556	Acidobacteria DA052 Ellin6513	No
	OTU1902	Alcaligenaceae	No
	OTU325	*Alicyclobacillus*	No
	OTU3160	Alphaproteobacteria Ellin329	No
	OTU2443	*Anaerococcus*	No
	OTU2474	*Anaerococcus*	No
	OTU1957	*Bacillus*	No
	OTU2874	Candidatus *Solibacter*	No
	OTU3668	*Cupriavidus*	No
	OTU884	*Enhydrobacter*	No
	OTU2972	Enterobacteriaceae	No
	OTU1033	Koribacteraceae	No
	OTU4038	Kouleothrixaceae	No
	OTU2970	Methylobacteriaceae	No
	OTU1824	*Methylobacterium*	No
	OTU333	*Methylobacterium komagatae*	No
	OTU3067	*Methylobacterium komagatae*	No
	OTU891	Microbacteriaceae	No
	OTU2409	*Paenibacillus*	No
	OTU2393	*Paenibacillus*	No
	OTU264	*Propionibacterium acnes*	No
	OTU2977	*Pseudomonas*	No
	OTU295	*Pseudomonas*	No
	OTU2956	*Pseudomonas*	No
	OTU2904	*Pseudomonas*	No
	OTU2437	*Rickettsiales mitochondria*	No
	OTU2882	*Rubrobacter*	No
	OTU2952	Solirubrobacterales	No
	OTU2386	*Sphingomonas*	No
	OTU1366	*Sphingomonas*	No
	OTU706	*Sphingomonas*	No
	OTU2396	*Stenotrophomonas geniculata*	No
	OTU2741	Acidobacteria DA052 Ellin6513	Yes
	OTU4033	Acidobacteriaceae	Yes
	OTU446	Actinomycetales	Yes

	OTU ID[a]	Taxonomy	Present as Singleton[b]
	OTU1847	Alphaproteobacteria Ellin329	Yes
	OTU3511	*Aquicella*	Yes
	OTU2852	Bacillaceae	Yes
	OTU1387	Bradyrhizobiaceae	Yes
	OTU2020	Candidatus *Solibacter*	Yes
	OTU3748	Candidatus *Solibacter*	Yes
	OTU1550	*Cyanobacteria* 4C0d-2 MLE1-12	Yes
	OTU3294	*Hyphomicrobium*	Yes
	OTU4189	Koribacteraceae	Yes
	OTU3105	Methylobacteriaceae	Yes
	OTU4170	*Propionibacterium acnes*	Yes
	OTU2435	Rhizobiales	Yes
	OTU4268	Rhizobiales	Yes
	OTU2401	*Sphingobium*	Yes
	OTU905	Sphingomonadaceae	Yes
Fungi			
	OTU706	Sordariomycetes	Yes
	OTU34	*Purpureocillium*	Yes

[a]OTU ID is a random number assigned during analysis. OTU numbers from the kitome analysis cannot be compared to other OTUs within this study or those identified in chapter 3.
[b]OTUs present as singletons were only identified in one sample and were successfully removed during the filtering process. Taxonomy is presented as the lowest possible rank that could be assigned to that OTU. Two or more OTUs with the same taxonomic assignment are genetically distinct and indicates that a lower taxonomic rank assignment was not available to differentiate between the OTUs.
[c]No archaea were identified in the kitome.

Table 3. Pairwise comparison of Shannon diversity indices for archaea/bacteria and fungal communities, grouped by treatment and sampling date. Comparisons were performed using nonparametric two-way t-tests.

	Factor 1	Factor 2	Factor 1 Shannon index[d] mean	Factor 1 Standard Deviation	Factor 2 Shannon index[d] mean	Factor 2 Standard Deviation	t stat	p-value
Treatment[a]								
Archaea/Bacteria[b]								
	0 kg N ha^{-1}	4.9 kg N ha^{-1} / 7 d	10.71	0.27	10.53	0.34	-0.85	1.00
	0 kg N ha^{-1}	4.9 kg N ha^{-1} / 14 d	10.71	0.27	10.83	0.12	-0.82	1.00
	0 kg N ha^{-1}	4.9 kg N ha^{-1} / 28 d	10.71	0.27	10.79	0.16	0.49	1.00
	0 kg N ha^{-1}	9.8 kg N ha^{-1} / 7 d	10.71	0.27	10.69	0.23	0.14	1.00
	4.9 kg N ha^{-1} / 7 d	4.9 kg N ha^{-1} / 14 d	10.53	0.34	10.83	0.12	-1.68	0.60
	4.9 kg N ha^{-1} / 7 d	4.9 kg N ha^{-1} / 28 d	10.53	0.34	10.79	0.16	1.38	1.00
	4.9 kg N ha^{-1} / 7 d	9.8 kg N ha^{-1} / 7 d	10.53	0.34	10.69	0.23	-0.77	1.00
	4.9 kg N ha^{-1} / 14 d	4.9 kg N ha^{-1} / 28 d	10.83	0.12	10.79	0.16	-0.44	1.00
	4.9 kg N ha^{-1} / 14 d	9.8 kg N ha^{-1} / 7 d	10.83	0.12	10.69	0.23	-1.11	1.00
	4.9 kg N ha^{-1} / 28 d	9.8 kg N ha^{-1} / 7 d	10.79	0.16	10.69	0.23	0.72	1.00
Fungi[c]								
	0 kg N ha^{-1}	4.9 kg N ha^{-1} / 7 d	7.86	0.51	7.80	0.36	-0.21	1.00
	0 kg N ha^{-1}	4.9 kg N ha^{-1} / 14 d	7.86	0.51	7.80	0.61	0.15	1.00
	0 kg N ha^{-1}	4.9 kg N ha^{-1} / 28 d	7.86	0.51	7.86	0.41	0.00	1.00
	0 kg N ha^{-1}	9.8 kg N ha^{-1} / 14 d	7.86	0.51	7.95	0.15	-0.34	1.00
	4.9 kg N ha^{-1} / 7 d	4.9 kg N ha^{-1} / 14 d	7.80	0.36	7.80	0.61	-0.02	1.00
	4.9 kg N ha^{-1} / 7 d	4.9 kg N ha^{-1} / 28 d	7.80	0.36	7.86	0.41	0.25	1.00
	4.9 kg N ha^{-1} / 7 d	9.8 kg N ha^{-1} / 7 d	7.80	0.36	7.95	0.15	-0.81	1.00
	4.9 kg N ha^{-1} / 14 d	4.9 kg N ha^{-1} / 28 d	7.80	0.61	7.86	0.41	0.17	1.00
	4.9 kg N ha^{-1} / 14 d	9.8 kg N ha^{-1} / 7 d	7.80	0.61	7.95	0.15	0.48	1.00
	4.9 kg N ha^{-1} / 28 d	9.8 kg N ha^{-1} / 7 d	7.95	0.15	7.95	0.15	-0.41	1.00
Sampling Date[e]								
Archaea/Bacteria[b]								
	11 June 2014	25 July 2014	10.46	0.34	10.72	0.13	-1.41	1.00
	11 June 2014	27 August 2014	10.46	0.34	10.83	0.14	-2.02	0.58
	11 June 2014	16 April 2015	10.46	0.34	10.60	0.19	-0.74	1.00
	11 June 2014	3 June 2015	10.46	0.34	10.93	0.11	2.60	0.03
	25 July 2014	27 August 2014	10.72	0.13	10.83	0.14	-1.20	1.00
	25 July 2014	16 April 2015	10.72	0.13	10.60	0.19	1.00	1.00
	25 July 2014	3 June 2015	10.72	0.13	10.93	0.11	2.42	0.52
	27 August 2014	16 April 2015	10.83	0.14	10.60	0.19	1.95	1.00
	27 August 2014	3 June 2015	10.83	0.14	10.93	0.11	1.08	1.00
	16 April 2015	3 June 2015	10.60	0.19	10.93	0.11	2.94	0.01
Fungi[c]								
	11 June 2014	25 July 2014	7.97	0.11	7.93	0.25	0.30	1.00

Factor 1	Factor 2	Factor 1 Shannon index[d] mean	Factor 1 Standard Deviation	Factor 2 Shannon index[d] mean	Factor 2 Standard Deviation	t stat	p-value
11 June 2014	27 August 2014	7.97	0.11	8.39	0.24	-3.10	0.41
11 June 2014	16 April 2015	7.97	0.11	7.34	0.45	2.71	0.27
11 June 2014	3 June 2015	7.97	0.11	7.64	0.18	-3.17	0.19
25 July 2014	27 August 2014	7.93	0.25	8.39	0.24	-2.60	0.35
25 July 2014	16 April 2015	7.93	0.25	7.34	0.45	2.28	0.53
25 July 2014	3 June 2015	7.93	0.25	7.64	0.18	-1.91	0.92
27 August 2014	16 April 2015	8.39	0.24	7.34	0.45	4.08	0.15
27 August 2014	3 June 2015	8.39	0.24	7.64	0.18	-4.99	0.15
16 April 2015	3 June 2015	7.34	0.45	7.64	0.18	1.21	1.00

[a]Treatments are reported as no nitrogen (N) or kilograms of N per hectare every 7, 14 or 28 days. N was applied as urea.
[b]Data from a depth of 1000 seqs/sample.
[c]Data from a depth of 4000 seqs/sample.
[d]Shannon Index as log base 2 using output from QIIME.
[e]Specific sampling dates

Table 4. Bray-Curtis dissimilarity matrix for archaea and bacteria, where 0.00 represents a completely identical microbial community and 1 represents a dissimilar community.

Year[a]			2015	2014	2014	2015	2015	2014	2014	2014	2014	2014	2014	2014	2014	2014	2014	2015	2015	2014	2015	2014	2015	2015	2014	2015	2015
	Month[b]		Apr	Aug	Aug	Jun	Jun	Jul	Jul	Jun	Jun	Jun	Jun	Jul	Jul	Aug	Aug	Jun	Apr	Apr	Aug	Apr	Jun	Apr	Jun	Jul	Jun	Jun
		Treatment[c]	9.8 kg N ha^{-1} / 7 d	No N	4.9 kg N ha^{-1} / 14 d	4.9 kg N ha^{-1} / 28 d	4.9 kg N ha^{-1} / 7 d	No N	4.9 kg N ha^{-1} / 7 d	No N	9.8 kg N ha^{-1} / 7 d	4.9 kg N ha^{-1} / 7 d	4.9 kg N ha^{-1} / 28 d	4.9 kg N ha^{-1} / 14 d	4.9 kg N ha^{-1} / 28 d	9.8 kg N ha^{-1} / 7 d	4.9 kg N ha^{-1} / 14 d	4.9 kg N ha^{-1} / 7 d	No N	4.9 kg N ha^{-1} / 7 d	4.9 kg N ha^{-1} / 28 d	4.9 kg N ha^{-1} / 28 d	4.9 kg N ha^{-1} / 14 d	9.8 kg N ha^{-1} / 7 d	9.8 kg N ha^{-1} / 7 d	No N	4.9 kg N ha^{-1} / 14 d
2015	Apr	9.8 kg N ha^{-1} / 7 d	0.00																								
2014	Aug	No N	0.72	0.00																							
2014	Aug	4.9 kg N ha^{-1} / 14 d	0.75	0.68	0.00																						
2015	Jun	4.9 kg N ha^{-1} / 28 d	0.77	0.73	0.78	0.00																					
2015	Jun	4.9 kg N ha^{-1} / 7 d	0.78	0.76	0.80	0.70	0.00																				
2014	Jul	No N	0.82	0.74	0.74	0.78	0.82	0.00																			
2014	Jul	4.9 kg N ha^{-1} / 7 d	0.76	0.72	0.71	0.78	0.79	0.73	0.00																		
2014	Jun	No N	0.81	0.79	0.84	0.81	0.83	0.84	0.82	0.00																	
2014	Jun	9.8 kg N ha^{-1} / 7 d	0.79	0.81	0.77	0.81	0.85	0.81	0.79	0.88	0.00																
2014	Jun	4.9 kg N ha^{-1} / 7 d	0.81	0.80	0.84	0.84	0.85	0.85	0.82	0.67	0.87	0.00															
2014	Jul	4.9 kg N ha^{-1} / 28 d	0.83	0.78	0.78	0.78	0.82	0.70	0.74	0.81	0.84	0.83	0.00														
2014	Jul	4.9 kg N ha^{-1} / 14 d	0.83	0.78	0.72	0.79	0.83	0.71	0.72	0.86	0.80	0.87	0.72	0.00													
2014	Aug	4.9 kg N ha^{-1} / 28 d	0.79	0.71	0.69	0.76	0.84	0.73	0.74	0.83	0.81	0.85	0.73	0.72	0.00												
2014	Aug	9.8 kg N ha^{-1} / 7 d	0.79	0.74	0.67	0.82	0.85	0.72	0.73	0.84	0.80	0.85	0.75	0.71	0.67	0.00											
2014	Jun	4.9 kg N ha^{-1} / 14 d	0.75	0.75	0.78	0.77	0.80	0.82	0.79	0.68	0.82	0.69	0.80	0.83	0.80	0.81	0.00										
2015	Apr	4.9 kg N ha^{-1} / 7 d	0.64	0.69	0.75	0.72	0.73	0.80	0.75	0.78	0.80	0.79	0.81	0.82	0.77	0.79	0.73	0.00									
2015	Apr	No N	0.69	0.69	0.72	0.72	0.73	0.77	0.73	0.80	0.78	0.81	0.78	0.78	0.75	0.76	0.76	0.67	0.00								
2014	Aug	4.9 kg N ha^{-1} / 7 d	0.82	0.79	0.72	0.85	0.86	0.82	0.79	0.91	0.79	0.91	0.86	0.80	0.79	0.75	0.87	0.83	0.81	0.00							
2015	Apr	4.9 kg N ha^{-1} / 28 d	0.74	0.74	0.74	0.74	0.79	0.79	0.78	0.86	0.77	0.87	0.80	0.78	0.74	0.78	0.81	0.71	0.69	0.79	0.00						
2014	Jun	4.9 kg N ha^{-1} / 28 d	0.74	0.73	0.76	0.76	0.78	0.80	0.76	0.68	0.82	0.70	0.79	0.82	0.78	0.78	0.64	0.71	0.73	0.86	0.79	0.00					
2015	Apr	4.9 kg N ha^{-1} / 14 d	0.69	0.69	0.74	0.70	0.73	0.79	0.76	0.77	0.81	0.78	0.78	0.80	0.75	0.80	0.72	0.65	0.66	0.84	0.67	0.71	0.00				
2015	Jun	9.8 kg N ha^{-1} / 7 d	0.74	0.73	0.79	0.66	0.68	0.82	0.78	0.81	0.81	0.83	0.83	0.83	0.81	0.83	0.76	0.71	0.70	0.85	0.77	0.76	0.71	0.00			
2014	Jul	9.8 kg N ha^{-1} / 7 d	0.69	0.64	0.69	0.71	0.71	0.78	0.70	0.78	0.78	0.78	0.79	0.78	0.75	0.76	0.72	0.69	0.70	0.79	0.77	0.71	0.70	0.68	0.00		
2015	Jun	No N	0.79	0.76	0.78	0.68	0.78	0.81	0.80	0.88	0.77	0.89	0.83	0.80	0.78	0.82	0.83	0.76	0.74	0.82	0.72	0.83	0.75	0.73	0.76	0.00	
2015	Jun	4.9 kg N ha^{-1} / 14 d	0.83	0.83	0.81	0.78	0.81	0.85	0.84	0.91	0.78	0.92	0.88	0.84	0.85	0.85	0.87	0.82	0.78	0.81	0.78	0.87	0.82	0.79	0.81	0.73	0.00

[a]Year Sample was collected.

[b]Sampling month, where Jun = 11 June for 2014 samples and 3 June for 2015 samples, Jul = 25 July 2014, Aug = 27 August 2014, Apr = 16 April 2015.

[c]Treatments are reported as no nitrogen (N) or kilograms of nitrogen per hectare every 7, 14 or 28 days. Treatments were applied from 7 May to 8 September 2014 and from 5 to 26 May 2015.

Table 5. Bray-Curtis dissimilarity matrix for fungi, where 0.00 represents a completely identical microbial community and 1 represents a dissimilar community.

Year[a]			2015	2015	2015	2015	2014	2014	2014	2014	2014	2014	2014	2014	2014	2014	2015	2014	2015	2014	2014	2014	2015	2015	2014	2015	2015
	Month[b]		Apr	Apr	Apr	Apr	Jul	Jun	Jun	Jul	Jun	Aug	Jul	Jul	Jun	Aug	Apr	Aug	Jun	Jul	Aug	Aug	Jun	Jun	Jun	Jun	Jun
		Treatment[c]	4.9 kg N ha-1 / 28 d	4.9 kg N ha-1 / 14 d	No N	9.8 kg N ha-1 / 7 d	4.9 kg N ha-1 / 28 d	4.9 kg N ha-1 / 14 d	No N	9.8 kg N ha-1 / 7 d	4.9 kg N ha-1 / 28 d	4.9 kg N ha-1 / 7 d	4.9 kg N ha-1 / 14 d	No N	4.9 kg N ha-1 / 7 d	No N	4.9 kg N ha-1 / 7 d	4.9 kg N ha-1 / 14 d	4.9 kg N ha-1 / 28 d	4.9 kg N ha-1 / 7 d	4.9 kg N ha-1 / 28 d	9.8 kg N ha-1 / 7 d	9.8 kg N ha-1 / 7 d	4.9 kg N ha-1 / 7 d	9.8 kg N ha-1 / 7 d	4.9 kg N ha-1 / 14 d	No N
2015	Apr	4.9 kg N ha-1 / 28 d	0.00																								
2015	Apr	4.9 kg N ha-1 / 14 d	0.54	0.00																							
2015	Apr	No N	0.39	0.43	0.00																						
2015	Apr	9.8 kg N ha-1 / 7 d	0.53	0.68	0.61	0.00																					
2014	Jul	4.9 kg N ha-1 / 28 d	0.68	0.76	0.71	0.75	0.00																				
2014	Jun	4.9 kg N ha-1 / 14 d	0.68	0.61	0.66	0.78	0.74	0.00																			
2014	Jun	No N	0.72	0.62	0.69	0.80	0.77	0.41	0.00																		
2014	Jul	9.8 kg N ha-1 / 7 d	0.58	0.61	0.58	0.68	0.63	0.57	0.59	0.00																	
2014	Jun	4.9 kg N ha-1 / 28 d	0.64	0.59	0.63	0.76	0.72	0.40	0.41	0.54	0.00																
2014	Aug	4.9 kg N ha-1 / 7 d	0.66	0.79	0.72	0.65	0.68	0.81	0.83	0.66	0.77	0.00															
2014	Jul	4.9 kg N ha-1 / 14 d	0.69	0.66	0.67	0.73	0.57	0.66	0.68	0.52	0.61	0.66	0.00														
2014	Jul	No N	0.74	0.67	0.70	0.76	0.57	0.69	0.68	0.57	0.63	0.72	0.47	0.00													
2014	Jun	4.9 kg N ha-1 / 7 d	0.68	0.66	0.68	0.78	0.75	0.36	0.40	0.60	0.40	0.81	0.67	0.72	0.00												
2014	Aug	No N	0.62	0.66	0.60	0.65	0.61	0.67	0.68	0.51	0.65	0.60	0.51	0.59	0.69	0.00											
2015	Apr	4.9 kg N ha-1 / 7 d	0.43	0.49	0.43	0.58	0.72	0.66	0.68	0.61	0.62	0.72	0.69	0.75	0.65	0.62	0.00										
2014	Aug	4.9 kg N ha-1 / 14 d	0.62	0.71	0.66	0.67	0.68	0.72	0.73	0.55	0.67	0.51	0.57	0.65	0.74	0.50	0.65	0.00									
2015	Jun	4.9 kg N ha-1 / 28 d	0.64	0.53	0.62	0.77	0.73	0.56	0.58	0.58	0.54	0.79	0.66	0.65	0.60	0.67	0.65	0.72	0.00								
2014	Jul	4.9 kg N ha-1 / 7 d	0.66	0.64	0.64	0.77	0.51	0.62	0.62	0.54	0.56	0.70	0.49	0.45	0.63	0.58	0.69	0.65	0.63	0.00							
2014	Aug	4.9 kg N ha-1 / 28 d	0.67	0.77	0.72	0.71	0.62	0.79	0.81	0.69	0.75	0.60	0.61	0.67	0.81	0.58	0.71	0.56	0.78	0.65	0.00						
2014	Aug	9.8 kg N ha-1 / 7 d	0.64	0.65	0.63	0.68	0.64	0.70	0.72	0.54	0.68	0.59	0.54	0.60	0.73	0.48	0.67	0.52	0.67	0.61	0.56	0.00					
2015	Jun	9.8 kg N ha-1 / 7 d	0.61	0.55	0.58	0.72	0.73	0.54	0.56	0.55	0.53	0.78	0.64	0.67	0.58	0.65	0.61	0.69	0.41	0.62	0.77	0.65	0.00				
2015	Jun	4.9 kg N ha-1 / 7 d	0.66	0.57	0.65	0.80	0.75	0.60	0.59	0.61	0.58	0.80	0.68	0.68	0.63	0.70	0.66	0.73	0.41	0.61	0.78	0.67	0.42	0.00			
2014	Jun	9.8 kg N ha-1 / 7 d	0.62	0.72	0.64	0.64	0.71	0.66	0.70	0.55	0.60	0.67	0.64	0.69	0.62	0.63	0.65	0.62	0.69	0.67	0.73	0.64	0.65	0.73	0.00		
2015	Jun	4.9 kg N ha-1 / 14 d	0.55	0.69	0.59	0.64	0.68	0.71	0.74	0.57	0.66	0.64	0.64	0.70	0.70	0.62	0.63	0.57	0.64	0.66	0.70	0.63	0.61	0.65	0.50	0.00	
2015	Jun	No N	0.54	0.64	0.53	0.67	0.70	0.64	0.68	0.54	0.61	0.69	0.62	0.68	0.67	0.58	0.61	0.62	0.54	0.63	0.71	0.60	0.52	0.60	0.55	0.48	0.00

[a]Year Sample was collected.

[b]Sampling month, where Jun = 11 June for 2014 samples and 3 June for 2015 samples, Jul = 25 July 2014, Aug = 27 August 2014, Apr = 16 April 2015.

[c]Treatments are reported as no nitrogen (N) or kilograms of nitrogen per hectare every 7, 14 or 28 days. Treatments were applied from 7 May to 8 September 2014 and from 5 to 26 May 2015.

Table 6. Archaeal/bacterial abundance averaged by sampling month. Taxonomic groups reflect the lowest possible assigned nomenclature.

		Sampling Date				
Taxonomic Groups		11 June 2014	25 July 2014	27 August 2014	16 April 2015	3 June 2015
Archaea	Unassigned Archaea	2.83E-04	5.19E-04	4.66E-04	4.51E-04	4.89E-04
	Parvarchaeota	3.12E-04	6.14E-05	1.21E-04	5.94E-05	8.40E-05
	Crenarchaeota	7.77E-03	3.68E-03	5.56E-03	4.31E-03	7.64E-03
	Euryarchaeota	1.48E-04	1.03E-03	1.40E-03	3.77E-04	3.75E-04
Bacteria	Unassigned Bacteria	3.75E-04	6.88E-04	4.92E-04	4.01E-04	4.66E-04
	Thermi	0	0	0	7.02E-06	1.48E-05
	Acidobacteria	6.32E-02	9.10E-02	7.33E-02	6.61E-02	9.39E-02
	Actinobacteria	5.54E-03	1.05E-02	6.06E-03	8.95E-03	1.15E-02
	Armatimonadetes	2.29E-04	4.00E-04	4.30E-04	3.28E-04	5.32E-04
	Bacteroidetes	2.03E-03	3.72E-03	4.01E-03	2.85E-03	6.33E-03
	BHI80-139	0	0	1.87E-05	0	0
	Chlorobi	3.59E-05	3.10E-04	1.42E-04	4.75E-05	1.25E-04
	Chloroflexi	6.77E-03	9.44E-03	8.15E-03	6.74E-03	1.20E-02
	Cyanobacteria	2.25E-03	2.79E-03	1.92E-03	4.20E-03	3.23E-03
	Elusimicrobia	5.24E-05	2.91E-04	2.47E-04	7.31E-05	1.18E-04
	FBP	1.03E-04	2.05E-04	2.27E-04	4.96E-05	2.80E-04
	FCPU426	4.34E-06	4.10E-05	8.58E-05	0	2.15E-05
	Fibrobacteres	6.77E-05	2.63E-04	3.84E-04	1.55E-04	3.57E-04
	Firmicutes	1.41E-04	2.74E-04	2.97E-04	8.47E-04	3.41E-04
	Gemmatimonadetes	7.17E-04	1.02E-03	1.09E-03	9.82E-04	8.04E-04
	GN02	1.78E-04	6.42E-04	5.88E-04	1.51E-04	2.93E-04
	Kazan-3B-28	8.69E-06	1.74E-05	0	0	0
	MVP-21	9.09E-05	1.06E-04	7.07E-05	1.71E-04	6.10E-05
	Nitrospirae	9.88E-05	2.29E-04	2.16E-04	3.70E-05	2.28E-04
	OD1	2.18E-03	7.20E-03	7.29E-03	2.63E-03	2.75E-03
	OP11	1.13E-02	4.76E-03	5.84E-03	6.45E-03	6.66E-03
	OP3	1.59E-05	6.77E-05	6.02E-05	0	0
	Planctomycetes	1.91E-03	5.37E-03	4.64E-03	2.58E-03	4.64E-03
	Proteobacteria	5.51E-02	1.02E-01	7.96E-02	7.00E-02	1.00E-01
	SBR1093	0	1.18E-05	0	0	6.75E-06
	Spirochaetes	4.68E-05	1.13E-04	7.07E-05	6.17E-06	5.00E-05
	SR1	2.39E-03	4.21E-05	4.73E-05	1.59E-04	4.39E-04
	Tenericutes	2.59E-05	2.68E-05	1.20E-05	3.21E-05	3.97E-05
	TM6	1.64E-04	8.34E-04	5.76E-04	2.05E-04	6.03E-04
	TM7	3.38E-03	7.51E-03	5.07E-03	8.66E-03	5.68E-03
	TPD-58	7.23E-06	0	1.20E-05	1.81E-05	6.82E-06
	Verrucomicrobia	9.92E-04	5.70E-03	4.79E-03	1.86E-03	2.46E-03
	WPS-2	1.35E-04	1.77E-04	1.90E-04	7.09E-05	2.17E-04

Table 7. Core microbiome[a] in the soil of annual bluegrass putting green turf receiving five rates of nitrogen and sampled from 11 June 2014 through 3 June 2015 in North Brunswick, NJ.

	OTU ID[b]	Taxonomic group
Archaea		
	OTU536	Cenarchaeales SAGMA-X
Bacteria		
	OTU0	Chloracidobacteria RB41 Ellin6075
	OTU10311	Bradyrhizobium
	OTU1045	Acidobacteriaceae
	OTU1066	Solibacterales
	OTU1139	Xanthomonadaceae
	OTU150	Koribacteraceae
	OTU1571	Alphaproteobacteria Ellin329
	OTU18773	Chloracidobacteria RB41 Ellin6075
	OTU2	Sinobacteraceae
	OTU204	OP11 OP11-3
	OTU2145	Chloracidobacteria DS-100
	OTU272	Candidatus Solibacter
	OTU322	Rhizobiales
	OTU3722	Rhizobiales
	OTU439	OP11 WCHB1-64d153
	OTU50	Acidobacteria DA052 Ellin6513
	OTU5170	Acidobacteria iii 1-832-20
	OTU5546	OD1 SM2F11
	OTU579	Alphaproteobacteria Ellin329
	OTU602	Rhizobiales
	OTU634	Bradyrhizobium
	OTU65	Rhizobiales
	OTU668	Acidobacteria DA052 Ellin6513
	OTU692	Rhodospirillaceae
	OTU72	Rhizobiales
	OTU726	Acidobacteria DA052 Ellin6513
	OTU768	Alphaproteobacteria
	OTU843	Hyphomicrobiaceae
	OTU951	Rhizobiales
	OTU99	Alphaproteobacteria
Fungi		
	OTU269	Myriangiales sp

[a]To be considered a member of the core microbiome, the OTU must be present in all samples. Taxonomic groups are presented as the lowest possible rank that could be assigned to that OTU. Two or more OTUs with the same taxonomic assignment are genetically distinct and indicates that a lower taxonomic rank assignment was not available to differentiate between the OTUs. Only OTUs that could be assigned to a taxonomic group are presented.

[b]OTU ID is a random number assigned during analysis. OTU numbers described for the core microbiome cannot be compared to those described in chapter 3.

Table 8. Bacteria phyla present in the soil of annual bluegrass putting green turf receiving five rates of nitrogen and sampled from 11 June 2014 through 3 June 2015 in North Brunswick, NJ. Phyla are in order of decreasing abundance.

Taxonomy	Average Abundance
Proteobacteria	8.14E-02
Acidobacteria	7.75E-02
Chloroflexi	8.62E-03
Actinobacteria	8.51E-03
OP11	7.00E-03
TM7	6.06E-03
OD1	4.41E-03
Planctomycetes	3.83E-03
Bacteroidetes	3.79E-03
Verrucomicrobia	3.16E-03
Cyanobacteria	2.88E-03
Gemmatimonadetes	9.23E-04
SR1	6.16E-04
Unidentified Bacterial Phyla	4.85E-04
TM6	4.76E-04
Armatimonadetes	3.84E-04
Firmicutes	3.80E-04
GN02	3.70E-04
Fibrobacteres	2.45E-04
FBP	1.73E-04
Nitrospirae	1.62E-04
WPS-2	1.58E-04
Elusimicrobia	1.56E-04
Chlorobi	1.32E-04
MVP-21	9.98E-05
Spirochaetes	5.73E-05
FCPU426	3.05E-05
OP3	2.88E-05
Tenericutes	2.73E-05
TPD-58	8.83E-06
Kazan-3B-28	5.21E-06
Thermi	4.36E-06
BHI80-139	3.75E-06
SBR1093	3.72E-06

Table 9. Bacterial genera identified in the soil of annual bluegrass putting green turf receiving five rates of nitrogen and sampled from 11 June 2014 through 3 June 2015 in North Brunswick, NJ. Candidate divisions are not included.

Acutodesmus	*Friedmanniella*	*Planctomyces*
Aerococcus	*Gemmata*	*Plesiocystis*
Afifella	*Gemmatimonas*	*Propionicimonas*
Agrobacterium	*Geobacter*	*Prosthecobacter*
Anaeromyxobacter	*Hyphomicrobium*	*Pseudomonas*
Aquicella	*Janthinobacterium*	*Pseudonocardia*
Asteroleplasma	*Kaistia*	*Ralstonia*
Asticcacaulis	*Kaistobacter*	*Ramlibacter*
Azospirillum	*Kineococcus*	*Rhizobium*
Bacillus	*Kouleothrix*	*Rhodoplanes*
Balneimonas	*Labrys*	*Rhodovastum*
Bdellovibrio	*Lautropia*	*Roseomonas*
Belnapia	*Legionella*	*Rubrivivax*
Blastomonas	*Leptolyngbya*	*Rudanella*
Bosea	*Limnohabitans*	*Sediminibacterium*
Bradyrhizobium	*Luteolibacter*	*Singulisphaera*
Brevibacterium	*Magnetospirillum*	*Sphingobium*
Burkholderia	*Mesorhizobium*	*Sphingomonas*
Candidatus *Koribacter*	*Methylibium*	*Sphingopyxis*
Candidatus *Liberibacter*	*Methylobacterium*	*Spirochaeta*
Candidatus *Solibacter*	*Methylopila*	*Spirosoma*
Candidatus *Xiphinematobacter*	*Methylosinus*	*Steroidobacter*
Caulobacter	*Microbacterium*	*Streptomyces*
Cellulomonas	*Microcoleus*	*Sulfuritalea*
Chitinophaga	*Microlunatus*	*Tatlockia*
Chryseobacterium	*Mogibacterium*	*Telmatospirillum*
Chthoniobacter	*Mycobacterium*	*Terriglobus*
Clostridium	*Mycoplana*	*Terrimonas*
Couchioplanes	*Nitrospira*	*Thermomonas*
Dechloromonas	*Nostoc*	*Uliginosibacterium*
Deinococcus	*Novosphingobium*	*Virgisporangium*
Desulfovibrio	*Ochrobactrum*	*Zoogloea*
Devosia	*Parvibaculum*	
Dokdonella	*Pasteuria*	
Dolichospermum	*Pedomicrobium*	
Exiguobacterium	*Pedosphaera*	
Fimbriimonas	*Phaeospirillum*	
Flavisolibacter	*Phenylobacterium*	
Flavobacterium	*Phormidium*	
Fluviicola	*Pilimelia*	
Frankia	*Pirellula*	

Table 10. Fungal abundance averaged by sampling month from the soil of annual bluegrass putting green turf receiving five rates of nitrogen and sampled from 11 June 2014 through 3 June 2015 in North Brunswick, NJ. Taxonomic groups reflect the lowest possible assigned nomenclature.

		Sampling Date				
		11 June 2014	25 July 2014	27 August 2014	16 April 2015	3 June 2015
Taxonomy						
Ascomycota	Myriangiales	1.92E-03	9.34E-04	7.91E-04	5.46E-04	5.22E-03
	Boliniales	1.76E-06	0	6.16E-06	2.66E-06	0
	Sordariomycetes	5.3E-06	0	0	0	0
Basidiomycota	Agaricales	2.14E-06	0	0	8.18E-06	0
Chytridiomycota	Chytridiomycota	2.14E-06	1.47E-05	1.02E-05	2.08E-05	1.29E-05
	Blastocladiella sp.	0	0	0	0	1.14E-05
	Chytridiomycota	0	0	0	6.75E-06	0
Glomeromycota	Paraglomerales	2.8E-06	0	6.16E-06	0	0
Rozellomycota	Rozellomycota	1.37E-04	1.66E-04	1.85E-04	1.28E-04	3.04E-05
Zygomycota	*Mortierella sp.*	0	0	0	1.08E-05	0
Unidentified Fungi	Unidentified Fungi	2.5E-06	4E-06	6.16E-06	0	0

Supplemental Table 1. Average monthly air temperature for New Brunswick, NJ. All temperatures are reported as degrees Celsius.

Sampling Month	Mean Temperature	Minimum Temperature	Maximum Temperature
June 2014	22	15	28
July 2014	24	17	28
August 2014	23	15	28
April 2015	11	4	17
June 2015	21	15	26

Supplemental Table 2. Soil Test results. Five soil cores were taken from each of the four replicated treatments and pooled. Results from each of the four replications are listed individually. Abbreviations and chemical symbols are as follows: LRI (lime requirement index), P (phosphorous), K (potassium), Ca (calcium), Mg (magnesium), B (boron), Zn (zinc), Mn (manganese), Cu (copper), and Fe (iron).

Month Collected	Treatment	pH	LRI	P lb/A	K lb/A	Ca lb/A	Mg lb/A	B ppm	Zn ppm	Mn ppm	Cu ppm	Fe ppm
July 2014	0.0 kg N ha^{-1}	5.62	7.63	63.1	154.8	909.6	156.4	1.658	10.140	69.340	2.745	135.200
	0.0 kg N ha^{-1}	5.47	7.69	50.6	175.0	860.6	156.8	1.826	10.500	52.390	2.464	140.200
	0.0 kg N ha^{-1}	5.59	7.73	41.7	148.3	710.2	121.4	2.307	8.946	53.620	2.077	92.170
	0.0 kg N ha^{-1}	5.57	7.77	49.4	171.1	903.4	165.8	2.078	11.830	64.150	4.515	122.100
	4.9 kg N ha^{-1} 28d	5.6	7.73	55.3	154.9	841.9	143.9	2.058	11.030	63.990	3.235	136.500
	4.9 kg N ha^{-1} 28d	5.9	7.71	38.0	127.1	739.6	136.4	1.385	7.460	51.040	2.024	94.130
	4.9 kg N ha^{-1} 28d	5.47	7.74	60.5	215.7	1096.8	188.7	2.303	13.260	79.310	3.036	121.700
	4.9 kg N ha^{-1} 28d	5.58	7.78	58.6	184.8	932.4	168.2	2.079	11.780	53.070	5.013	144.400
	4.9 kg N ha-1 14d	5.78	7.7	42.2	118.5	752.4	136.1	1.427	8.192	49.120	3.003	95.310
	4.9 kg N ha-1 14d	5.6	7.83	31.3	121.4	646.1	119.0	1.935	7.384	45.240	2.079	101.500
	4.9 kg N ha-1 14d	5.64	7.78	76.4	232.6	1240.8	211.8	1.746	14.860	98.280	4.553	177.100
	4.9 kg N ha-1 14d	5.59	7.79	46.8	133.1	833.6	144.6	2.042	9.461	52.190	3.569	134.900
	4.9 kg N ha-1 7d	5.63	7.71	52.0	137.8	791.0	143.2	1.851	10.180	64.420	2.571	154.700
	4.9 kg N ha-1 7d	5.66	7.74	56.7	165.3	895.7	156.4	1.509	10.570	64.790	2.593	131.500
	4.9 kg N ha-1 7d	5.5	7.74	46.1	148.4	856.9	148.9	1.911	9.903	62.490	3.178	154.200
	4.9 kg N ha-1 7d	5.41	7.76	50.1	133.0	950.2	156.9	2.093	10.890	71.900	3.655	127.300
	9.8 kg N ha-1 7d	5.53	7.73	51.2	180.8	1091.0	182.5	1.669	14.200	88.990	3.440	162.400
	9.8 kg N ha-1 7d	5.68	7.74	50.7	192.8	990.7	170.5	2.109	11.650	83.400	2.636	138.000
	9.8 kg N ha-1 7d	5.62	7.74	48.2	136.4	873.4	147.9	1.929	10.580	67.480	2.620	116.600
	9.8 kg N ha-1 7d	5.57	7.72	50.4	160.2	901.7	157.2	1.879	11.460	71.800	4.352	129.900
August 2014	0.0 kg N ha^{-1}	5.74	7.79	50.6	119.3	910.5	153.9	2.021	10.010	54.750	2.867	108.000
	0.0 kg N ha^{-1}	5.68	7.55	44.8	130.9	812.4	147.3	2.029	8.465	33.240	2.928	100.700
	0.0 kg N ha^{-1}	5.79	7.67	54.0	137.7	912.8	153.2	2.093	10.240	61.910	3.036	99.370
	0.0 kg N ha^{-1}	5.52	7.73	50.3	145.5	939.0	164.4	1.413	10.420	41.710	3.226	99.860
	4.9 kg N ha^{-1} 28d	5.64	7.34	47.8	128.1	789.4	142.5	1.908	9.272	43.370	3.162	109.600
	4.9 kg N ha^{-1} 28d	5.81	7.61	61.7	131.4	1150.8	198.1	2.108	10.870	68.480	3.561	123.900
	4.9 kg N ha^{-1} 28d	5.73	7.58	53.3	151.0	1022.3	171.8	2.196	11.340	56.010	3.219	100.900
	4.9 kg N ha^{-1} 28d	5.76	7.74	36.4	100.1	697.0	120.5	2.178	7.329	21.060	2.600	66.990
	4.9 kg N ha-1 14d	5.85	7.46	49.9	97.2	874.3	150.6	1.989	8.631	36.050	3.491	96.020
	4.9 kg N ha-1 14d	5.82	7.62	40.5	103.6	742.8	127.9	1.912	8.006	36.990	2.728	99.940
	4.9 kg N ha-1 14d	5.77	7.67	55.4	130.1	968.7	161.9	1.875	10.390	59.830	3.205	106.200
	4.9 kg N ha-1 14d	5.59	7.65	44.3	123.1	879.1	151.2	1.810	8.991	40.410	2.872	111.600
	4.9 kg N ha-1 7d	5.59	7.42	44.7	102.2	718.9	132.4	1.822	8.958	34.690	3.236	103.900
	4.9 kg N ha-1 7d	5.75	7.62	70.8	147.7	1060.4	174.6	1.917	12.100	60.610	3.683	127.400
	4.9 kg N ha-1 7d	5.59	7.69	45.0	123.7	1050.2	174.6	1.895	11.810	47.080	3.657	105.800
	4.9 kg N ha-1 7d	5.64	7.74	42.9	100.7	868.3	142.3	2.100	8.831	38.170	3.078	80.330
	9.8 kg N ha-1 7d	5.66	7.46	38.7	99.9	867.8	144.0	2.103	10.180	49.000	3.109	95.950
	9.8 kg N ha-1 7d	5.76	7.56	52.1	139.5	1167.9	206.3	2.067	11.960	70.560	4.090	123.700
	9.8 kg N ha-1 7d	5.65	7.71	49.0	108.9	935.6	153.7	1.929	10.800	55.260	3.590	99.060

Month Collected	Treatment	pH	LRI	P lb/A	K lb/A	Ca lb/A	Mg lb/A	B ppm	Zn ppm	Mn ppm	Cu ppm	Fe ppm
	9.8 kg N ha-1 7d	5.76	7.76	40.4	106.1	771.6	127.6	1.445	8.293	43.560	2.928	88.910
June 2015	0.0 kg N ha^{-1}	6.28	7.18	39.5	73.3	805.5	114.5	1.35	9.05	39.01	1.87	70.73
	0.0 kg N ha^{-1}	6.22	7.4	52.9	80.0	907.6	143.2	1.72	12.52	41.20	2.92	111.40
	0.0 kg N ha^{-1}	6.11	7.49	65.5	100.1	1050.4	154.3	1.70	12.20	48.45	2.72	107.60
	0.0 kg N ha^{-1}	5.99	7.62	45.4	90.4	924.7	136.7	1.89	11.83	48.93	2.43	82.44
	4.9 kg N ha^{-1} 28d	5.92	7.64	33.6	57.2	729.3	106.4	2.13	8.74	40.60	2.01	71.35
	4.9 kg N ha-1 28d	6.04	7.71	44.6	102.9	863.7	134.9	1.84	8.96	43.98	2.39	105.00
	4.9 kg N ha-1 28d	5.91	7.7	39.4	89.7	873.6	128.0	2.19	10.65	45.55	2.51	77.44
	4.9 kg N ha-1 28d	6.11	7.76	47.7	93.2	1440.9	153.6	1.54	19.65	60.36	2.52	101.80
	4.9 kg N ha-1 14d	6.03	7.76	44.8	84.0	948.0	134.3	1.88	9.75	43.74	1.99	73.59
	4.9 kg N ha-1 14d	5.82	7.76	38.7	88.3	893.6	130.7	2.14	9.64	39.22	2.05	85.47
	4.9 kg N ha-1 14d	5.92	7.81	45.1	97.9	972.9	142.9	1.99	11.08	55.44	2.45	94.00
	4.9 kg N ha-1 14d	5.69	7.82	36.4	77.8	803.8	117.1	1.90	8.71	35.94	1.70	81.95
	4.9 kg N ha-1 7d	5.75	7.84	36.7	71.1	701.1	103.0	2.35	8.65	34.76	2.27	64.43
	4.9 kg N ha-1 7d	5.7	7.79	47.5	76.4	766.1	116.2	2.26	9.74	49.47	2.56	90.23
	4.9 kg N ha-1 7d	5.95	7.86	37.9	65.1	782.5	112.1	2.18	8.97	37.02	2.24	63.92
	4.9 kg N ha-1 7d	5.79	7.82	44.4	88.3	798.2	125.0	2.38	9.87	47.45	2.50	81.57
	9.8 kg N ha-1 7d	5.73	7.8	38.8	72.7	753.7	109.2	2.00	9.16	44.19	2.47	81.72
	9.8 kg N ha-1 7d	5.63	7.85	37.3	81.0	622.5	105.3	2.30	8.23	35.78	2.39	51.10
	9.8 kg N ha-1 7d	5.73	7.86	47.0	98.6	729.1	124.7	2.13	9.62	54.90	2.36	73.60
	9.8 kg N ha-1 7d	5.83	7.84	39.8	60.3	651.7	102.5	2.03	8.29	39.99	2.10	65.11

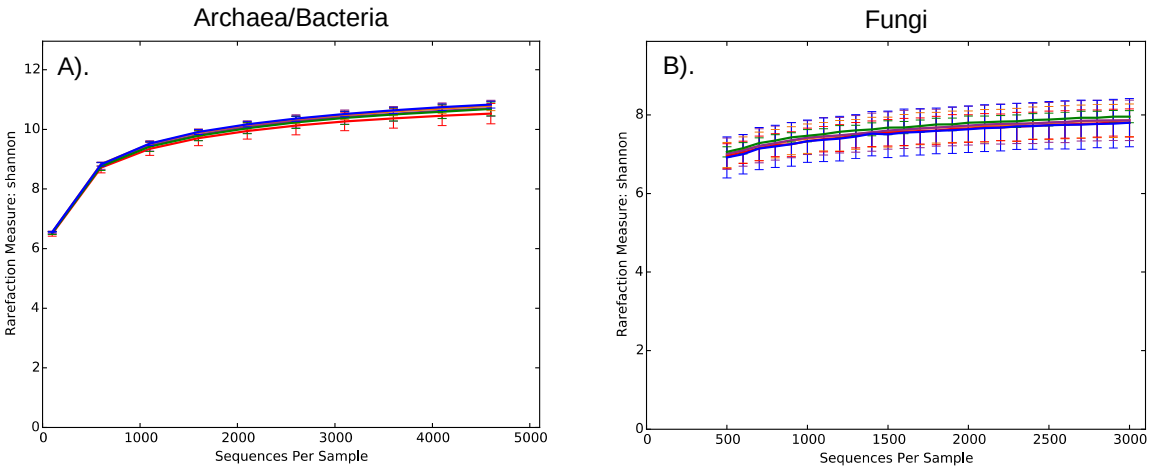

Figure 1. Rarefactons curves for archaea and bacteria across treatments and sampling dates. For archaea/bacteria (1A) and fungi (1B), rarefaction curves plateau, demonstrating microbial diversity has been adequately captured in our samples. Key to treatments is as follows: purple line = 0 kg N ha^{-1}, red line = 4.9 kg N ha^{-1} every 7 d, blue line = 4.9 kg N ha^{-1} every 14 d, orange line = 4.9 kg N ha^{-1} every 28 d, and green line = 9.8 kg N ha^{-1} every 7 days. Treatments were applied from 7 May to 8 September 2014 and on 5, 12, 19, and 26 May 2015.

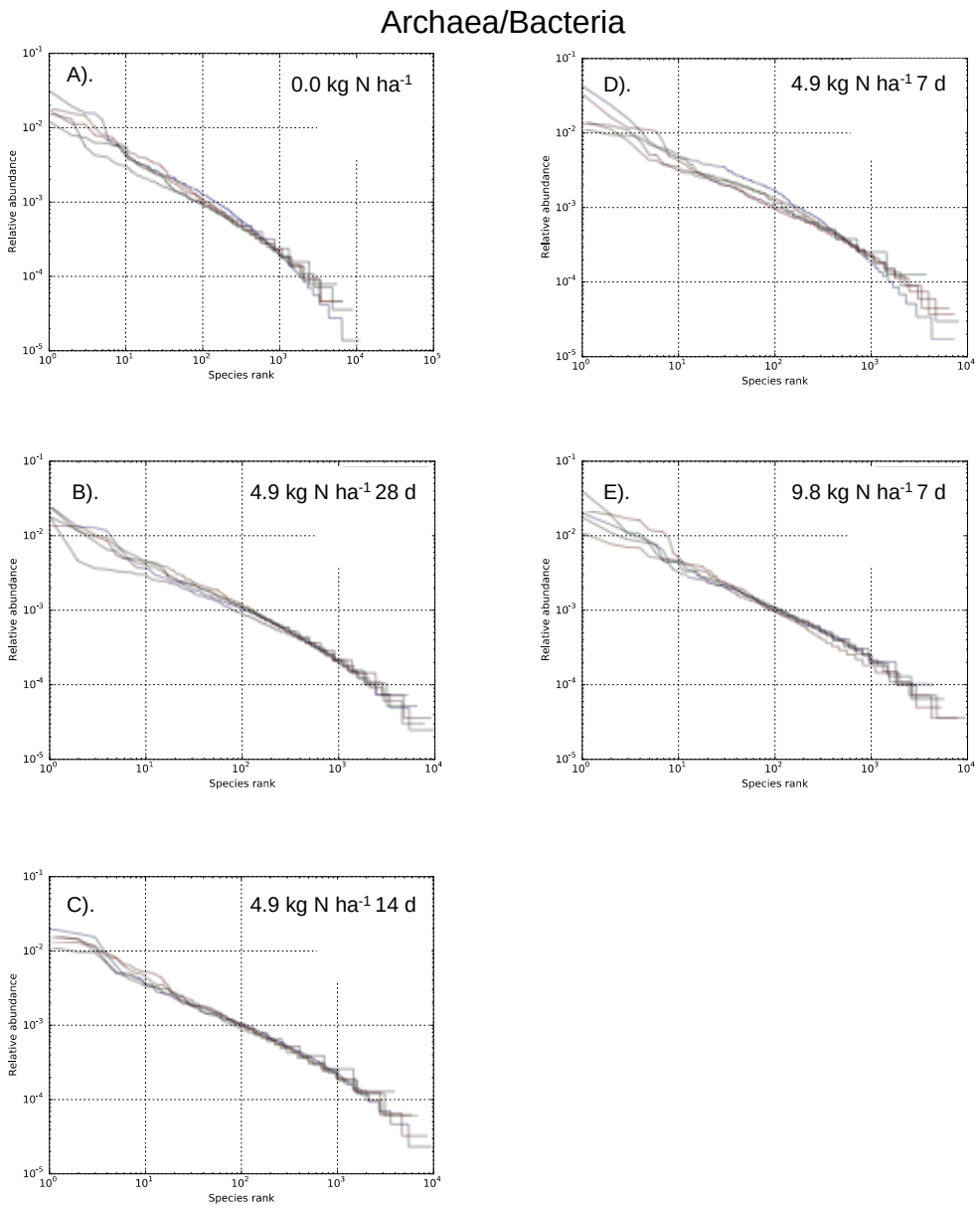

Figure 2. Rank abundance plots of archaea/bacteria communities. Species are plotted on the x-axis and relative abundance are plotted on the y-axis. Species rarity increases moving right along the x-axis. Each line depicts a sampling month where 16 samples were pooled. Sampling months are as follows: red line = 16 April 2015, blue line = 11 June 2014, orange line = 25 July 2014, green line = 27 August 2014, and purple line = 3 June 2015. A). 0.0 kg N ha^{-1}, B). 4.9 kg N ha^{-1} every 28 d, C). 4.9 kg N ha^{-1} every 14 d D). 4.9 kg N ha^{-1} every 7 d, and E). 9.8 kg N ha^{-1} every 7 d. Treatments were applied from 7 May to 8 September 2014 and on 5, 12, 19, and 26 May 2015.

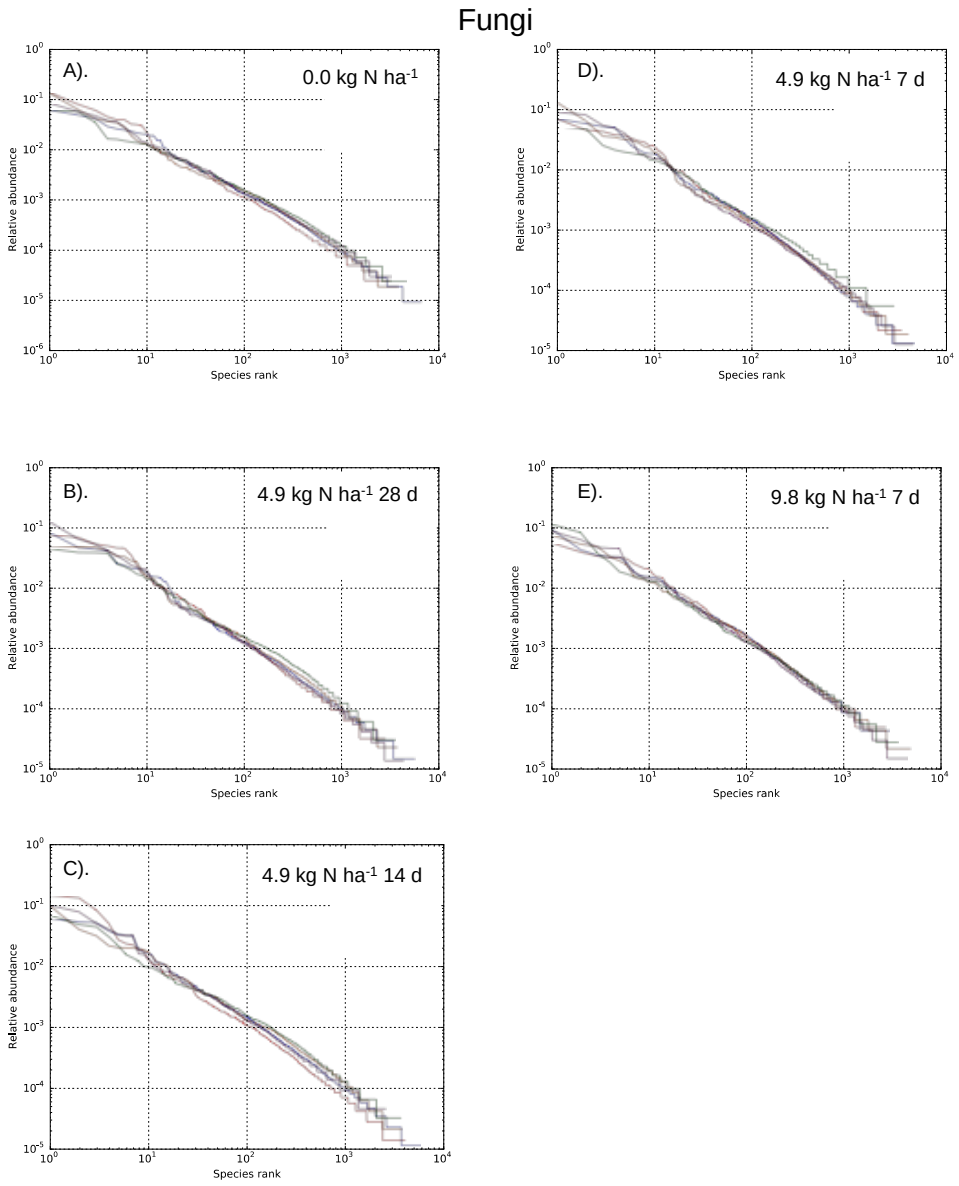

Figure 3. Rank abundance plots of fungal communities. Samples are divided by treatments. Species are plotted on the x-axis and relative abundance are plotted on the y-axis. Species rarity increases moving right along the x-axis. Each line depicts a sampling month where 16 samples were pooled. Sampling months are as follows: red line = 16 April 2015, blue line = 11 June 2014, orange line = 25July 2014, green line = 27 August 2014, and purple line = 3 June 2015. A). 0.0 kg N ha^{-1}, B). 4.9 kg N ha^{-1} every 28 d, C). 4.9 kg N ha^{-1} every 14 d D). 4.9 kg N ha^{-1} every 7 d, and E). 9.8 kg N ha^{-1} every 7 d. Treatments were applied from 7 May to 8 September 2014 and on 5, 12, 19, and 26 May 2015.

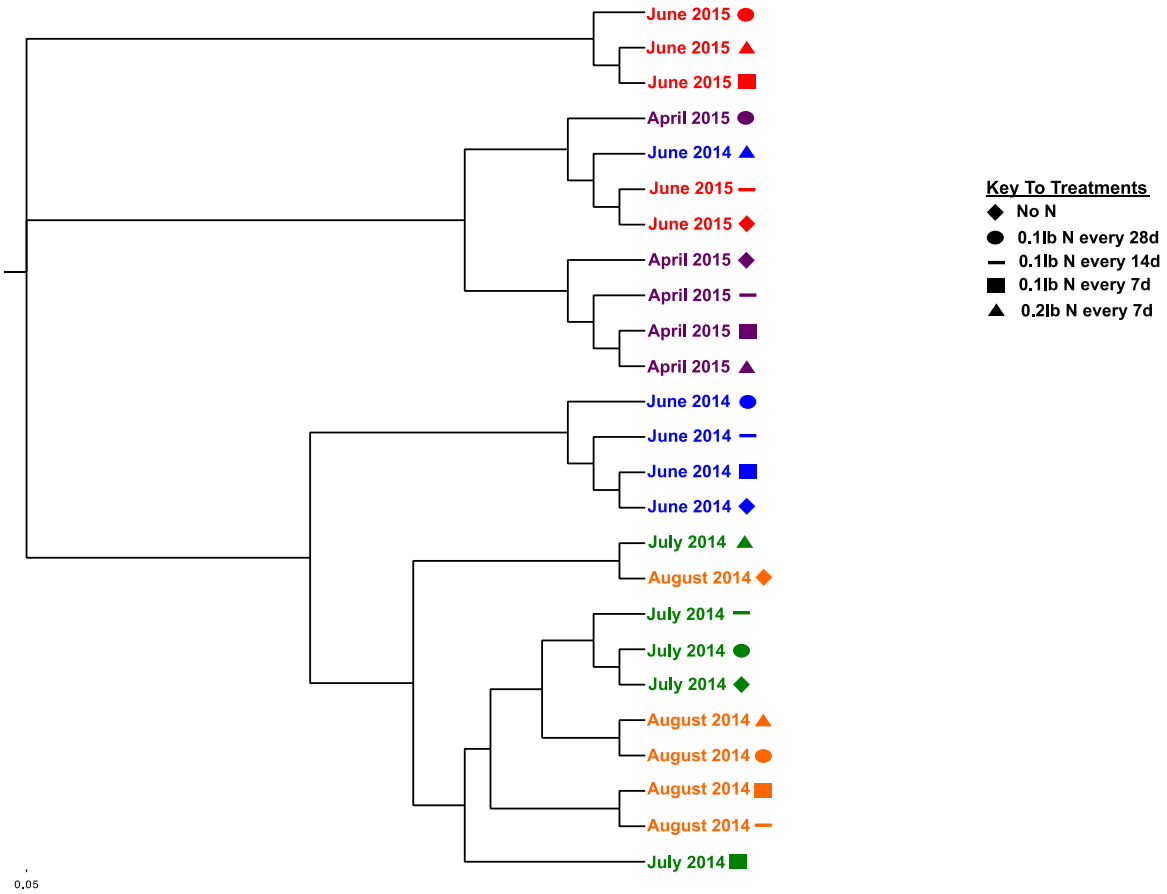

Figure 4. Neighbor joining tree generated from Bray Curtis dissimilarity matrix for archaea/bacteria. Samples are divided by nitrogen treatments and sampling date.

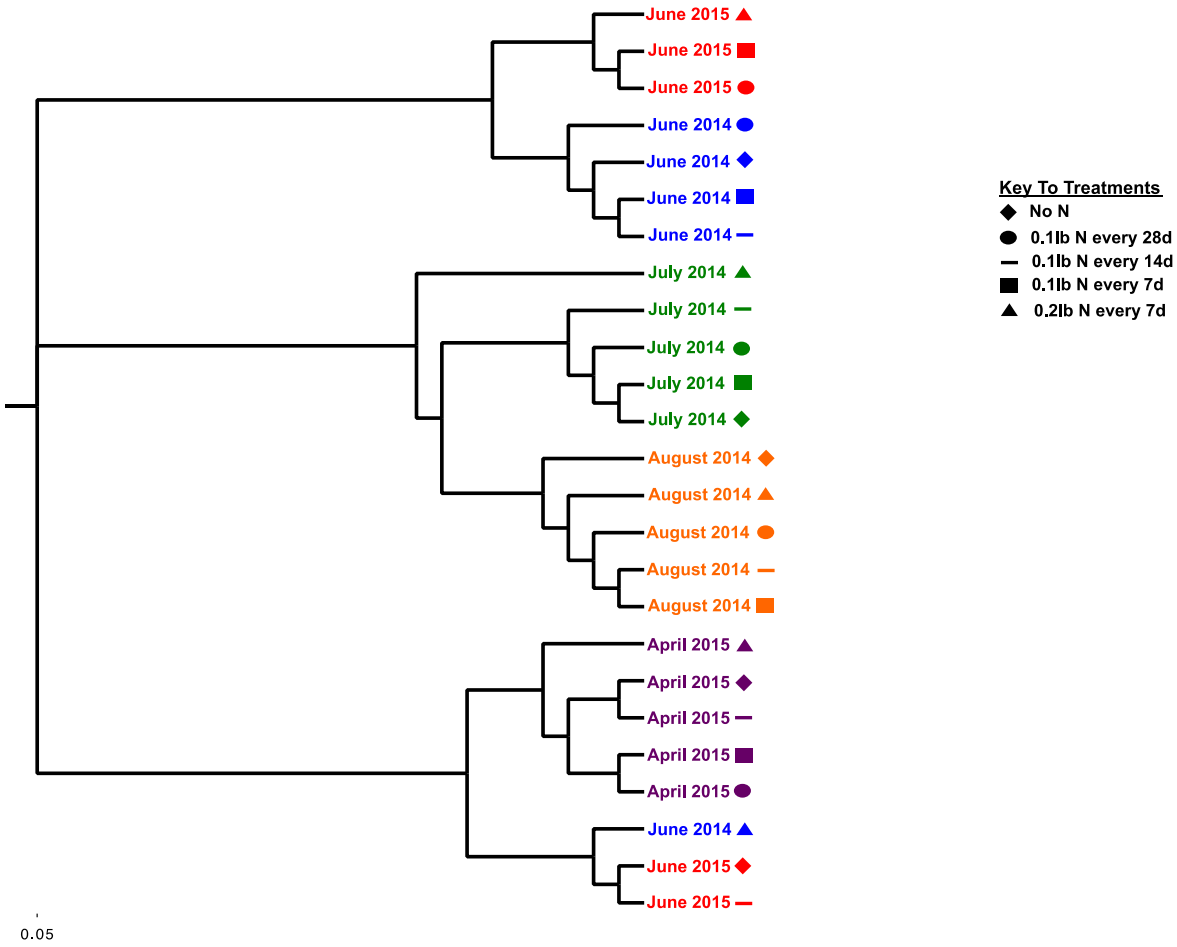

Figure 5. Neighbor joining tree generated from Bray Curtis dissimilarity matrix for fungi. Samples are divided by nitrogen treatments and sampling date.

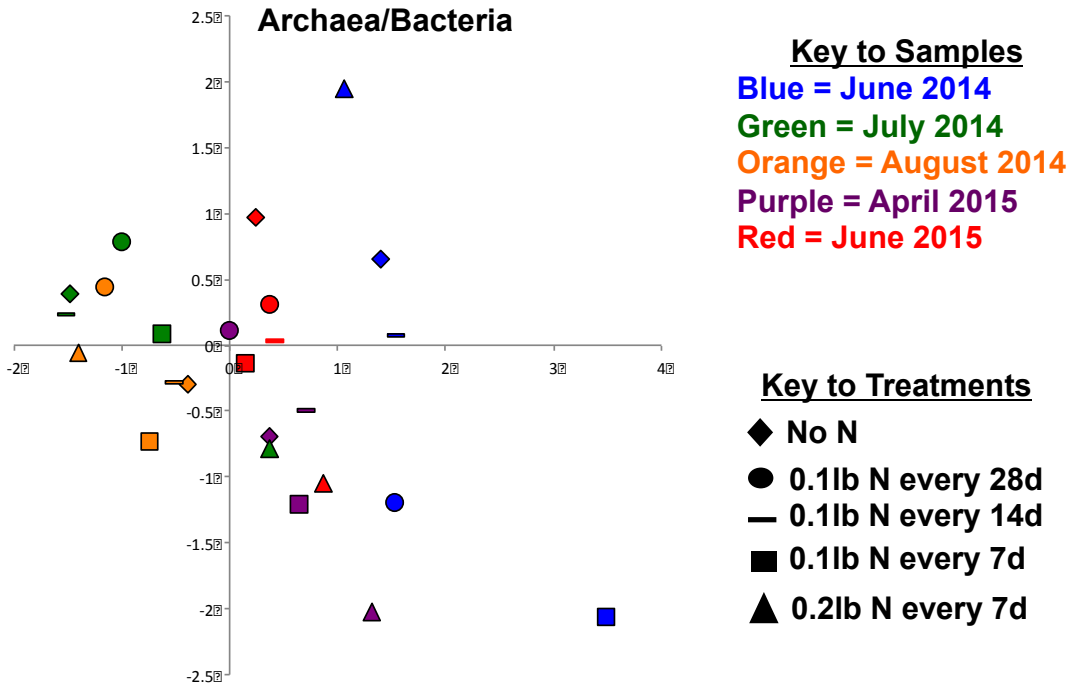

Figure 6. Multivariate detrended correspondence analysis of archaea/bacteria communities across 25 sample sites. Analyses are based on genus level OTU assignments.

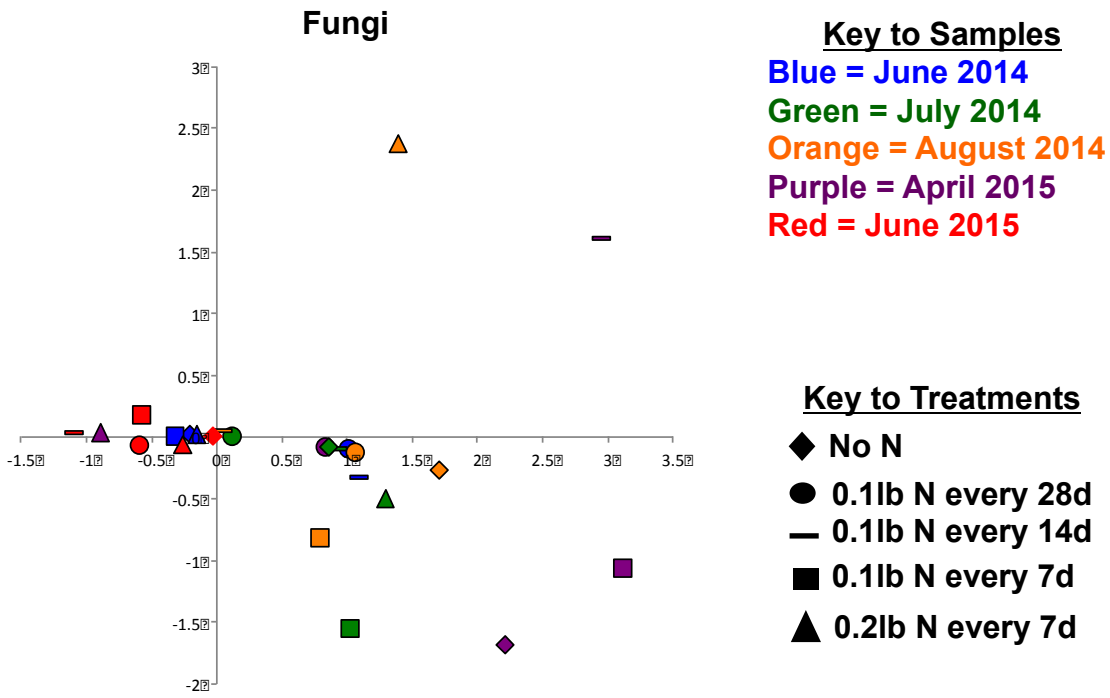

Figure 7. Multivariate detrended correspondence analysis of fungal communities across 25 sample sites. Analyses are based on genus level OTU assignments.

CHAPTER 3: Metagenomic analysis of the soil microbial community in *Poa annua* turf receiving different fertility treatments reveals unexpected and widespread diversity

ABSTRACT

Golf course putting greens are intensively managed environments that require frequent applications of fertility to maintain healthy turfgrass. Nutrients like nitrogen (N) and potassium (K) are commonly applied, yet little is known about whether they affect pathogenic or beneficial microorganisms in the rhizosphere. N is known to affect disease expression, thus it was hypothesized that this is related to impact on the rhizosphere microbial community. In this study, the objective was to examine the impact of high-input N and K fertility regimes on the composition, diversity and distribution of archaea, bacteria and fungal communities in the soil of annual bluegrass (ABG, *Poa annua*) putting green turf. Soil was sampled from two field studies entitled field 1K or field 2N. Field 1K received either 132 kg N ha^{-1} yr^{-1}, 200 kg K_2O ha^{-1} yr^{-1}, or 132 kg N ha^{-1} yr^{-1} + 200 kg K_2O ha^{-1} yr^{-1}, and field 2N received 100 or 200 kg N ha^{-1} yr^{-1}, where N was applied as urea [$CO(NH_2)_2$] and K as KCl. Three 15.9 mm x 50.8 mm soil cores were sampled from four replicated plots of each fertility treatments for a total of 60 samples. Multiplexed next-generation Illumina sequencing of the ITS (fungi) and 16s (archaea, bacteria) regions generated 2.3 x 10^7 paired-end reads. The QIIME pipeline picked 8.3 X 10^5 operational taxonomic units (OTUs), 4.1% of which were identified as archaea, 62.1% bacteria and 30.1% fungi. Microbial diversity was high across both studies sampled, regardless of treatment. Presence/absence analysis revealed no significant OTUs, however simulated power analyses indicated that significance would likely have

been detected with more sampling ($n \geq 20$). Differences in microbial community abundance were apparent across all fertility treatments for archaea, bacteria and fungi. In both field studies, 20.5% of the total archaea/bacteria and 23.5% of total fungi were present in differential abundance across all treatments. In general, K treatments and plots receiving low rates of N increased microbial abundance. For example, the archaea clone SAGMA-X, the only 16s OTU present in abundance >1% of all samples in the field 1K study and a species that metabolizes urea, was highest in abundance in the K only treatment; whereas, mycorrhizal fungi in the Glomeromycota were most abundant in plots receiving the N+K (dield 1K) and the low N treatment (dield 2N). Detrended correspondence analysis revealed samples clustering by study site, but not by treatment, indicating that other management practices or underlying soil fertility may also be contributing to the community dynamics observed in this system. These data show that N and K can influence the microorganisms inhabiting the soil of annual bluegrass turf, and that this environment is very diverse and species-rich.

INTRODUCTION

Turfgrasses are ubiquitous components of parks, roadsides, home lawns, athletic fields and golf courses around the world. They provide many benefits to humans and ecosystems, such as pollution absorption, erosion control, bioremediation, temperature moderation and increased human health and wellness when used as recreational surfaces (Stier et al. 2013). Like any man-made agronomic system, the turfgrass system may also yield potentially negative environmental impacts if not carefully managed. Some turfgrass ecosystems (*e.g.* golf courses) require high levels of water, pesticides and fertilizer inputs to maintain their aesthetic and recreational value (Gange et al. 2003). For example, putting greens receive daily mowing and irrigation as well as frequent pesticide and fertility inputs to maintain acceptable turfgrass quality and playability (ball roll distance; green speed) (Breuninger et al. 2013, Frank and Guertal 2013). Fertility programs on putting greens usually include regular applications of nitrogen (N) and potassium (K), the two nutrients required in the highest quantities in the turfgrass system (Frank and Guertal 2013a, 2013b). These frequent fertilizer applications, combined with the sandy structure of many putting greens, can lead to an increased potential for nutrient leaching (Erickson et al. 2001, Lee et al. 2003, Petrovic 2004, Frank et al. 2006, Mangiafico and Guillard 2006, Erickson et al. 2008). Fertilizer inputs in turfgrass systems have been the subject of environmental concern, as nutrient leaching and runoff have been implicated in other agronomic systems as contributing to soil and water pollution, marine eutrophication, increased greenhouse gas emissions, decreased carbon sequestration and loss of biodiversity (Follett 2001, Mulvaney et al. 2009, Vitousek et al. 1997). However, when fertilizers are applied at recommended rates for the specific

turfgrass species, studies have shown that little nutrient leaching occurs in established turfgrass stands (see Breuninger et al. 2013, Frank and Guertal 2013a, 2013b). As a result, such turfgrass systems are often subjected to intense public scrutiny (Wheeler and Nauright 2006, Briassoulis 2010). In particular, the general public may perceive putting greens in a negative manner, since these are the most intensely managed areas on golf courses.

While understanding the long-term fate of N and K applied to golf course putting greens turf is important for ecosystem preservation, a vital constituent of the putting green environment is often overlooked – the impact of these nutrients on the resident rhizosphere microbial community. Microbial communities are capable of acclimating to changing soil nutrient content, and have been shown to vary in species composition based on changing soil environments in agricultural systems (van Diepeningen 2006), however, little is known about the impact of frequent applications of N and K on the rhizosphere microbial communities on golf course putting greens. Nitrogen fertilization has been reported to reduce microbial biomass (Trededer 2008) and alter fungal species composition and decrease bacterial diversity in boreal and forest ecosystems (Wallenstein et al. 2006, Allison et al. 2007). In grasslands, Leff et al. (2015) found applications of N (applied as time-release urea over four years) to decrease mycorrhizae in the Glomeromycota and increase Archaea in the Crenarchaeota and bacteria in the Alphaproteobacteria and Actinobacteria, but these environments may not be comparable to those of a man-made system such as golf course putting greens. Moreover, few studies have examined the impact of K fertilization on microbial communities, though soil K levels optimal for plant growth have been associated with higher plant diversity (Janssens

et al. 1998). Healthy soils are often associated with a diverse number of organisms capable of contributing to nutrient cycling, as well as organic matter and plant health and productivity (Arias et al. 2005) although the abundance of different functional groups may also have an impact. Thus, assessing species diversity and the composition, distribution and abundance of organisms in the rhizosphere of putting green turf may provide an indication of the relative heath and productivity of this high-input ecosystem.

Several studies have quantified microbial species diversity in turfgrass putting greens (Bigelow et al. 2002, Elliot et al. 2004, Elliot et al. 2008) and some have examined the effect of N on these populations using culture-based approaches (Mancino et al. 1993, Elliot and des Jardin 1999, Elliot et al. 2003). For example, on creeping bentgrass (*Agrostis stolonifera*) putting greens in Arizona, Mancino et al. (1993) examined microbial populations in experimental plots receiving either no fertilizer, a water-soluble N source (21:7:14), or a water insoluble N source that contained additional microbial inoculants (Greens Restore N; 47C-6N). There was no significant difference in bacterial counts in any of the treatments, but both N sources slightly increased fungal counts (Mancino et al. 1993). However, the nutritional make up of each product tested varied. Natural organic N sources increased counts of one bacterium (*Stenotrophomonas. maltophilia*) in a bermudagrass putting green (*Cynodon dactylon*) on one date in Florida; however, the organic N treatments utilized were derived from two very different sources - sewage sludge and a combination of plant, blood, and bone meal (Elliot and des Jardin 1999). Thus, these treatments also contained varying levels of other nutrients in an undefined manner, making it impossible to determine the effect of N alone on the microbial community (Elliot and des Jardin 1999). In order to assess the impact of N on

microbial communities in bermudagrass and creeping bentgrass putting green turf, Elliot et al. (2008) examined two rates of urea (260 kg N ha^{-1} yr^{-1} versus 520 kg N ha^{-1} yr^{-1}) at several sites in the southeastern U.S.A. where turf has an extended growing season, and found the higher rate of N increased bacterial counts on several sampling dates using culture-based techniques.

Although culturing methods have worked well for quantifying overall counts of Gram-negative bacteria, for Gram-positive bacteria, fluorescent pseudomonads, and actinomycetes, plating on selective media has rarely yielded definitive species identifications and cannot be used to assess the effect of N on the vast population of unculturable microbes (Mancino et al. 1993, Elliot and des Jardin 1999, Bigelow et al. 2002, Elliot et al. 2004). Culturing methods can underestimate microbial diversity from soils (Kent and Triplett 2002). For example, studies have estimated that only 0.1% to 1% of soil bacteria can actually be cultured (Torsvik et al. 1990, Amann et al. 1995, Torsvik and Ovreas 2002). Phospholipid fatty acid (PLFA) profiles have also been used to assess total microbial biomass in turfgrass putting greens and have shown that putting green construction can influence bacterial populations in the rhizosphere, with temporary putting greens exhibiting very different PLFA profiles then established (mature) putting greens (Bartlett et al. 2007). However, PFLA analysis does have limitations, as the presence of a particular PLFA profile cannot always be linked to a specific microorganism, and generally cannot be used for a species-level identification (Hill et al. 2007). Thus, previous studies have only captured a small subset of the total microbial community in the turfgrass rhizosphere, and have provided little information about the identity of the microorganisms impacted by management practices.

Molecular technologies circumvent many of the issues associated with culture- and biochemical-based technologies, and PCR-based approaches have most recently become the standard for analyzing microbial species in soil (Kent and Triplett 2002, Tringe et al. 2005). In particular, next generation sequencing of the collective cohort of microbial genomes or diagnostic amplicons, such as the 16s rDNA or internal transcribed spacer region, directly from the environment has provided insight into the diversity present in soil ecosystems, and just how little is known about the inhabitants of the soil (Riesenfeld et al. 2004). Such techniques have only recently been applied to golf course putting greens (see chapter 2), and, as a result, there is only general information regarding microbial community structure. In particular, the soil of annual bluegrass putting greens have been shown to contain a wide array of microorganisms, several of which were identified as beneficial microbes (*Pseudomonas, Burkholderia, Glomeromycota,* etc.) or species potentially involved in the breakdown of urea (archaea SAGMA-X; chapter 2). However, there is no information on what impact, if any, the addition of different levels of nutrients such as N and K have on the abundance or presence of microorganisms in the turfgrass rhizosphere, and if these applications negatively impact the important species described above. Thus, the objective of this study was to determine the composition, diversity and distribution of archaea, bacteria and fungi in the soil of annual bluegrass (*Poa annua*) putting green turf receiving high-input applications of N and K. More specifically, the impact of different levels of N and K fertility regimes on overall microbial diversity and abundance, and whether fertility treatments might potentially be used to select for a favorable rhizosphere community to improve turfgrass health and disease resistance in the future were examined.

MATERIALS & METHODS

Experimental Plots

Two separate field trials of annual bluegrass turfgrass grown on a Nixon sandy loam (fine-loamy, mixed, mesic Type Hapludaults) and maintained as putting green turf were sampled for this study. Both field trials were initiated in 2012 in North Brunswick, New Jersey, U.S.A. to examine the impacts of potassium and/or nitrogen fertility on the development of anthracnose disease caused by the fungus *Colletotrichum cereale* (Inguagiato et al. 2008, Schmid et al. 2013). The first trial consisted of research plots receiving different potassium (K) treatments with or without nitrogen (henceforth referred to as the Field 1K study), and the second trial consisted of research plots receiving two different nitrogen (N) treatments (henceforth referred to as the Field 2N study). At the time of sampling, both studies were at the end of their second year of fertility applications. Annual bluegrass is one of the most commonly managed grasses on golf course putting greens (Mao and Huff 2012) in the northeastern U.S.A., making it an ideal host environment to sample the soil microbial community.

For the Field 1K study, plots were arranged in a randomized complete block with four replications. Each plot measured 1.8 m by 1.8 m. Plots were mowed daily with a triplex putting green mower (model 3100, Toro Co., Bloomington, MN) bench-set at 2.8-mm. Treatments in the potassium trial included (1) N alone (132 kg N ha^{-1} yr^{-1}), (2) K alone (200 kg K_2O ha^{-1} yr^{-1}), or (3) a combined treatment of N and K (132 kg N ha^{-1} yr^{-1} + 200 kg K_2O ha^{-1} yr^{-1}, 1:1, N+K molar-adjusted ratio) and were applied every 14 days beginning 23 April through 8 November 2013 (16 applications) at a rate of 4.9 and 13.7 kg ha^{-1} for N and K, respectively. An additional N application was made to all plots on 26 April at a rate of 23 kg N ha^{-1}. At the time of sample collection (11 September 2013),

total N and K applied during the growing season was 132 kg N ha^{-1}, 166 kg K ha^{-1}, respectively. In 2012, N and K treatments were applied at the same rates, except N and K were applied weekly from 25 April to 9 May 2012 (3 applications). Biweekly applications began 22 May and ended 6 November 2012. The nitrogen and potassium sources in the Field 1K study were urea ($CO(NH_2)_2$) and potassium chloride (KCl), respectively.

For the Field 2N study, plots were arranged in a 2 x 2 x 6 factorial using a split-split-plot experimental design with four replications. The treatment factors include: two levels of mowing height (3.2 mm and 2.3 mm), two levels of N fertility (4.9 kg N ha^{-1} of urea every 7 or 14 days), and six fungicide programs designed to surpress anthracnose disease. Only plots mowed at 3.2 mm and receiving no fungicides for anthracnose control were sampled. Each plot measured 1.8 m by 1.8 m. Plots were mown daily using a walking greens mower (model 2100, Toro Co., Bloomington, MN) with a bench set mowing height of 3.2-mm. Nitrogen applications included either (1) a low rate of N (100 kg N ha^{-1} yr^{-1}), or (2) a high rate of N (200 kg N ha^{-1} yr^{-1}), applied as 4.9 kg N ha^{-1} of urea every 14 or 7 days, respectively, from 9 April to 1 October 2012 and 8 April to 30 September 2013. On 19 March and 8 October 2012 and 18 March and 7 October 2013, the low N and high N programs also received 18.3 kg N ha^{-1} as a 1.14:1 combination of water-soluble (urea) and slow-release (methylene urea); the high N program received a second application of the same rate and material on 2 April and 22 October 2012 and 1 April and 21 October 2013.

General Field Maintenance

Both field studies received overhead irrigation that was supplemented by hand watering with a syringe hose to maintain moderately dry conditions typical of golf course putting greens in the northeastern U.S.A. Topdressing was applied as kiln-dried, medium-coarse, silica sand every 14 days at rates adjusted to match the growth of the turf canopy. Selected fungicides were applied to both studies on a preventative basis as broadcast applications to suppress fungal diseases such as dollar spot (caused by *Sclerotinia homoeocarpa*), brown ring patch (caused by *Waitea circinata*), brown patch (caused by *Rhizoctonia solani*), and summer patch (caused by *Magnaporthiopsis poae*) using products that have been shown to be ineffective against anthracnose disease (Towers et al. 2002). Specifically, dollar spot was controlled with vinclozolin [3-(3, 5-dichlorophenyl)-5-ethenyl-5-methyl-2, 4-oxazolidinedione] at 1.5 kg a.i. ha^{-1} or boscalid {3-pyridinecarboxamide, 2-chloro-N-[4'-chloro(1,1'-biphenyl)-2-yl]} at 0.4 kg a.i. ha^{-1} every 14 d from 13 May to 15 August 2013, brown ring patch with flutolanil [N-(3-isopropoxyphenyl)-2-(trifluoromethyl)benzamide] at 6.4 kg a.i ha^{-1} every 14 d from 4 April to 1 May 2013, brown patch with flutolanil at 6.4 kg a.i. ha^{-1} every 14 d from 1 May to 23 August 2013, and summer patch with azoxystrobin [methyl (E)-2-{2-[6-(2-cyanophenoxy) pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate] at 3 kg a.i. ha^{-1} every 28 d from 17 May to 7 August 2013. One application of chlorothalonil (tetrachloroisophthalonitrile) at 12.6 3 kg a.i. ha^{-1} was applied at the end of the season on 3 September 2013 to control algae and promote anthracnose recovery.

Moss was controlled on both studies with broadcast applications of carfentrazone-ethyl {ethyl 2-chloro-3-[2-chloro-5-[4-(difluoromethyl)-3-methyl-5-oxo-1,2,4-triazol-1-yl]-4-fluorophenyl]propanoate} at 0.03 kg a.i ha^{-1} on 14 May, 15 June, 4 July, 15 July,

and 5 August 2013. The seed head suppressant ethephon [(2-chloroethyl) phosphonic acid] at 3.3 kg a.i. ha^{-1} and the vegetative suppressant trinexapac-ethyl [4-(cyclopropyl-α-hydroxy-methylene)-3,5-dioxocyclohexanecarboxylic acid ethylester] at 0.05 kg a.i. ha^{-1} were applied on 15 March, and 3 and 17 April 2013, followed by weekly applications of trinexapac-ethyl from 24 April to 21 November 2013. Chlorantraniliprole {3-bromo-N-[4-chloro-2-methyl-6-[(methylamino)carbonyl]phenyl]-1-(3-chloro-2-pyridinyl)-1H-pyrazole-5-carboxamide) at 0.15 kg a.i. ha^{-1} and bifenthrin {2-methyl-3-phenylphenyl)methyl (1*S*,3*S*)-3-[(*Z*)-2-chloro-3,3,3-trifluoroprop-1-enyl]- 2,2-dimethylcyclopropane-1-carboxylate} at 0.12 kg a.i. ha^{-1} were applied on 1 May and 30 June 2013, respectively, to control annual bluegrass weevils [*Listronotus maculicollis* (Kirby)]. Applications of ethephon, trinexapac-ethyl, and chlorantraniliprole were applied as broadcast applications to all plots in both studies.

Soil Samples

Experimental treatments for both studies were last applied 1 wk prior to the sampling date (11 September 2013). No pesticides were sprayed at least 19 days prior to sampling, except for chlorothalonil, which was applied eight days prior to sampling on 3 September 2013 on both study sites to promote recovery from anthracnose disease. Soil was sampled by taking three 15.9 mm diameter x 50.8 mm deep soil cores from four replicated plots of each of the three treatments in the Field 1K study and two treatments in the Field 2N study, for a total of 60 samples. This sampling depth contains the many fibrous roots of annual bluegrass. The roots are spatially distributed throughout the sample core. As such, the entire core is in the sphere of influence from the roots (Hartmann et al. 2008), and will be referred to as the rhizosphere henceforth. All plots

were sampled randomly within a 30 cm by 45 cm region located in the center of the 1.8 m by 1.8 m plots. Cores were immediately placed on ice following removal. Prior to DNA extractions, soil samples were individually screened through a 2.5 mm sieve to remove any plant matter, debris or large soil particulates.

DNA Extractions, PCR and Library Preparation

Genomic DNA was extracted using the PowerSoil DNA Isolation Kit (Mo-Bio, Carlsbad, CA) and PCR amplification of the organism-specific gDNA ribosomal markers from archaea/bacteria (16s) and fungi was conducted as previously described (see chapter 2).

Next generation sequencing libraries were prepared from the pooled amplicons for each of the 60 samples using the Nextera Index Kit (Illumina, San Diego, CA), with identifying indices and Illumina adapters attached via a 12 cycle PCR to each sample to allow all 60 samples to be multiplexed. Libraries were quantified using the QIAxcel System (QIAGEN, Gaithersburg, MD) and Qubit fluorometer (Life Technologies, Grand Island, NY), normalized to 4 nM, and pooled into a single sample. One percent phiX (Illumina) and two indexed gDNA libraries of the fungus *C. cereale* were pooled into the same sequence run to increase library complexity and serve as controls. Following denaturing, the pooled libraries were diluted to 20 pM and sequenced on Illumina's MiSeq platform using a 600 cycle MiSeq v3 Reagent Kit (Illumina, San Diego, CA).

Data Analysis

For all analyses, the Field 1K study and the Field 2N study were analyzed separately, as was 16s and ITS data. Data were processed using the QIIME pipeline (version 1.8; Caporaso et al. 2010) on an Amazon EC2 image (Amazon machine image

number: ami-e788a08e). Reads were assembled using fastq-join in the ea-utils package (Aronesty 2011), only if there were no base pair differences in the overlap region. Joined reads were demultiplexed using the split_libraries_fastq.py script. Sequences with ambiguous bases or a quality score < 20 were removed from analysis. After filtering, 16s and ITS reads were separated using custom programming scripts in LabVIEW (National Instruments, Austin, TX). Chimeric reads were removed using USEARCH (Edgar et al. 2011). Operational taxonomic units (OTUs) were identified using a *de novo* OTU picking approach in UCLUST (Edgar 2010) with a 97% similarity cutoff. Singletons were removed and excluded from future analysis. Identification of archaea and bacteria was determined using the RDP Classifier 2.2 (Wang et al. 2007) and the Greengenes reference database version 12_10 (McDonald et al. 2012, Werner et al. 2012). Fungal identification was determined using the BLAST algorithm (Altschul et al. 1990) and the UNITE + INSDC reference database (Abarenkov et al. 2010). A custom database composed of ITS sequence data from fungal and oomycete pathogens of turfgrass was compiled from published sequence data and inserted into QIIME to detect the presence of common pathogens of annual bluegrass. Taxonomic identification was determined using the RDP Classifier 2.2 (Wang et al. 2007).

OTUs without matches in the Greengenes or UNITE +INSDC database were filtered from the OTU file using custom C++ parsing scripts. Any OTUs < 200 bp were removed from the OTU file and the remaining OTUs were BLASTed against the nucleotide database in GenBank using Standalone BLAST (Tao 2010).

All nomenclature is presented as the lowest taxonomic rank that was assigned for all analyses.

Statistical Analyses

Community analyses. The Vegan package in R was used for detrended correspondence analysis. Archaea and bacteria were rarified to a depth of 1000 in 100 step increments with 10 replicates. For fungal ITS samples, rarefactions were performed at a depth of 4000 in 100 step increments with 10 replicates. All rarefactions were performed in QIIME. Rarefaction curves were generated to determine if the microbial community reached saturation with the samples taken in this study. Alpha diversity metrics were calculated in QIIME using the Shannon index and compared using a nonparametric two-way t-test, where Monte Carlo permutations of the dataset were used to calculate the p-value. Rank abundance plots were generated for each sample to display relative species abundance using QIIME.

Microbial diversity and community analyses were generated using OTU abundance data.

Presence-absence testing. For each study, OTUs were identified in each sample and differences in presence/absence of microorganisms between fertility treatments was determined with paired- comparisons using a 2 x 2 Fisher's exact test in R. P-values were adjusted using the Benjamini-Hochberg FDR to correct for the possibility that differences were observed by chance. P-values less than 0.05 were considered significant. A statistical power simulation analysis was conducted to determine how the presence/absence of OTUs between treatments would change with increased replication (more samples) per treatment using R. This was completed by maintaining the proportion of observed sample counts in the 2 x 2 comparisons and increasing the sample size (n) for each treatment by 2. Values of n examined ranged from 14 (the original

sample size 12 x 2) to 50. Once the sample size was artificially increased, a 2 x 2 Fisher's exact test was performed on the simulated data.

Differential abundance testing. Differential abundance of microorganisms was assessed between fertility treatments. Traditionally, nonparametric methods, such as Mann-Whitney or Kruskall-Wallis tests, have been used to assess differential abundance among OTUs (Weiss et al. 2015). However, these tests are not always appropriate. For example, when OTU counts are normalized to account for variations in sequencing depth (e.g. rarefactions), the resulting abundance data is no longer an independent variable, thus violating this assumption of such tests (Fagerland and Sandvik 2009, Kurtz et al. 2015, Weiss et al. 2015). Furthermore, tests such as Kruskall-Wallis assume that the observations in each group come from sample distributions of the same shape, thus it may give inaccurate results if this assumption is violated (Fagerland and Sandvik 2009). Recently, Kruskall-Wallis tests have been shown to only perform well with datasets that do not contain many zeros (Paulson et al. 2013a). Since the data sets in this study contained an abundance of zeros, and for the other reasons mentioned above, MetagenomeSeq (Paulson et al. 2013b) was used to assess differential abundance in both studies. MetagenomeSeq is an analytical tool, available in the Bioconductor package, and implemented in R and is used to analyze differential abundance in sparse datasets like those generated from environmental marker surveys (Paulson et al. 2013b). Specifically, this method accounts for datasets with multiple zeros, or OTUs that are absent from a large number of samples. Data was normalized with cumulative-sum scaling (CSS), a method where OTU counts are divided by the cumulative sum of counts, up to the *qth* quantile determined by the data (Paulson et al. 2013b), to account for

varying depths of sequencing coverage in the dataset. This technique has been shown to work well with environmental marker-gene data (Paulson et al. 2013b). Following CSS normalization, a zero-inflated Gaussian model was implemented to accurately model the proportion of zero-count OTUs, and hence, most accurately estimate the abundance of OTUs that exhibited positive counts. OTUs with less than five positive samples were excluded from analysis (Paulson et al. 2013b).

Data Availability

All sequence data has been deposited at the NCBI's Sequence Read Archive and is available under accession number SRP044292. All supplemental data tables are available from www.eden.rutgers.edu/~lbeirn/dissertation.

RESULTS

Sequence Data

Illumina sequencing of the ribosomal amplicons across 60 samples generated 2.8 x 10^7 reads, 2.3 x 10^7 of which passed quality filtering. After read stitching and demultiplexing, an average of 1.38 x 10^5 sequences per sample were generated, with an average read length of 253 bp. From all sequences, 66,805 (1.1% of the total sequences) were identified as chimeras and removed from the dataset.

Overall Microbial Community Analysis

In total, 8.3 X 10^5 OTUs were identified from the 60 sample sites, 7.3 X 10^5 of which represented archaea/bacteria and 9.0 X 10^4 were fungi. Of these OTUs, 58% were identified as singletons and removed from analysis. Archaea, bacteria and fungi were identified from all samples. On average, 4.1% of the sequences were archaeal genera, 26.7% of the sequences were bacterial genera and 29.7% of the sequences were fungal genera. The remaining 39.5% of sequences were not identified to the OTU (genus) level with the Greengenes or UNITE +INSDC databases. From the unidentified sequences, BLAST searches identified 89.5% as sharing similarity to uncultured bacterial clones, 0.16% sharing similarity with uncultured archaea clones and 1.4% sharing similarity to unidentified fungal clones. Plant DNA, amplified from the fungal primers, represented 0.09% of the sequences unassigned to OTUs. The remaining 8.9% of the unassigned sequences could not be identified by BLAST.

For all microorganisms, detrended correspondence analysis (DCA) showed the 36 samples from the Field 1K study clustered separately from the 24 samples of the Field 2N

study (Figures 4 A and B). There was no overlap of samples between the Field 1K study and the Field 2N study, thus both studies were analyzed separately.

Presence/Absence Analyses

For the Field 1K study, 181343 archaea/bacteria and 46562 fungal OTUs were identified. OTU counts ranged from 0 to 38193 for archaea/bacteria and from 0 to 117727 for fungi in the Field 1K study. In the Field 2N study, 298874 archaea/bacteria and 23365 fungal OTUs were identified. OTU counts ranged from 0 to 55135 for archaea/bacteria and from 0 to 49283 for fungi in the Field 2N study. For each field study, tables were generated that contained a list of each OTU identified with its corresponding taxonomy, along with a count of the number of times the OTU was observed from a given sample (Supplemental Tables 1-2 Field 1K study, 3-4 Field 2N study). Visual examination of the count table showed that a large number of zeros were present in the dataset. For example, in just the first 25 archaea/bacteria OTUs of the Field 1K study, 818 zero count OTUs were observed, compared to just 82 non-zero count OTUs across all 36 samples in the study. These sporadic presence/absence data are consistent with environmental survey data (Paulson et al. 2013b), and could mean that either the absent OTUs were not detected due to an insufficiency in sequence depth of coverage, or that these OTUs represent rare taxa and may not have been captured in all samples.

For both the Field 1K and the Field 2N study, paired comparisons between treatments using the 2 x 2 Fisher's test revealed no OTUs exhibiting differential presence or absence associated with any fertility treatments (Benjamini-Hochberg FDR corrected $p = 1$). However, raw (pre-corrected) p values for ~1% of total OTUs were < 0.05,

suggesting there may have been true biological significance in the dataset, but that this significance could not be detected with the current sample size (n = 12 / treatment) or the statistical test employed. To test whether sample size played a role in this observation, 2 x 2 Fisher's tests were run using simulated datasets exhibiting the same OTU counts, but larger sample sizes (n = 14-50). These power simulation analyses revealed that as n increased, FDR corrected p values began to drop below the level of significance at p = 0.05 (Table 1). In particular, when simulated data was analyzed with n = 20, OTUs began to display treatment associated presence and absences at significant levels. As sample size increased, even more OTUs exhibited treatment associated presence and absence differences at statistically significant levels (Table 1).

Field 1K Study: Microbial Diversity and Community Composition

Rank abundance plots showed similar richness and evenness among samples for both archaea/bacteria and fungi in the Field 1K study (Figure 1A-F). Only for more rare species (further right on the x-axis), did the slopes for some samples flatten out prematurely. However, rarefaction analysis curves for all microbial groups reached a plateau (Figure 2A-D).

Alpha diversity calculated using the Shannon index is summarized in Table 2. Nonparametric two-way t-tests showed no significant difference in alpha diversity between the three treatments for either archaea/bacteria or fungi (p = 0.55 – 1.0; Table 2). Overall, archaea/bacteria diversity was higher than fungal diversity, regardless of treatment (p = 0.05).

In total, three archaea phyla and 46 bacterial phyla were identified from the Field 1K study. For archaea, total abundance averaged across all Field 1K samples was as

follows: Crenarchaeota 13%, Parvarchaeota 0.4%, and Euryarchaeota 0.4%. For bacteria, total abundance averaged across all Field 1K samples was as follows: Proteobacteria 17.7%, Acidobacteria 17%, OP11 4.8%, Chloroflexi 3.4%, Planctomycetes 2.2%, Bacteroidetes 1.7%, Actinobacteria 1.6%, and OD1 1.1%. The remaining 38 bacterial phyla were present in abundance <1%. A complete list of archaeal and bacterial taxa identified in the Field 1K study is presented in Supplemental Table 5.

In total, seven fungal phyla and one fungal-like phyla (Ichtyosporea) were identified from the Field 1K study. Total abundance averaged across all Field 1K samples was as follows: Ascomycota 39.85%, unidentified fungal genus 12.28%, Basidiomycota 3.05%, and Glomeromycota 1.13%. The remaining phyla were present in abundance <1%. A complete list of fungal taxa identified is presented in Supplemental Table 5.

Field 1K Study: Differential Abundance

All fertility treatments altered the abundance of archaea and bacteria communities (Supplemental Table 6), especially for Acidobacteria and Proteobacteria, the two most common bacterial groups identified in this study. Due to the large nature of Supplemental Table 6, the following data is presented in a summarized form below. When comparing N treated plots to plots treated with N+K, 12245 OTUs were differentially abundant, but represented only 7% of the total archaea/bacteria population in the Field 1K study (Supplemental Table 6, First Tab). The following number of OTUs in dominant phyla (phyla in abundance > 1%) were affected: 3093 Proteobacteria, 2828 Acidobacteria, 388 Chloroflexi, 300 Crenarchaeota, 285 candidate division OP11, 234

Planctomycetes, 3093 Proteobacteria, 82 candidate bacterial division TM7, 76 Gemmatimonadetes ,and 44 Verrucomicrobia.

When comparing K to N treated plots, 11190 archaea/bacteria OTUs were differentially abundant, accounting for 6% of the total population (Supplemental Table 6, Second Tab). The following number of OTUs in dominant phyla (phyla in abundance > 1%) were affected: 3340 Acidobacteria OTUs, 3073 Proteobacteria, 461 Chloroflexi, 407 Planctomycetes, 363 candidate division OP11, 289 Crenarchaeota, 217 Actinobacteria, 165 Bacteroidetes, 152 Actinobacteria, 148 Bacteroidetes, 132 candidate division TM7, and 107 Verrucomicrobia.

When comparing plots treated with K to N+K treated plots, 9691 OTUs were differentially abundant, representing just 5% of the total archaea/bacteria population (Supplemental Table 6, Third Tab). The following number of OTUs in dominant phyla (phyla in abundance > 1%) were affected: 3838 Acidobacteria, 3098 Proteobacteria, 556 Planctomycetes, 498 Chloroflexi, 460 candidate bacterial division OP11, 384 Crenarchaeota, 306 Actinobacteria, 192 Bacteroidetes, 171 Verrucomicrobia, and 140 candidate bacterial division TM7.

In general, K treatments increased OTU counts compared to N and N+K treated plots. Specific organisms that were always more abundant in K treated plots include: the Crenarchaeota clone SAGMA-X, Acidobacteria, Alphaproteobacteria, and Xanthomonadaceae.

Fertility treatments also altered the abundance of fungal OTUs in the Field 1K study (Supplemental Table 7), especially for Ascomycetes, the most common fungal group identified in this study. When comparing N treated plots to turf treated with N+K,

6486 OTUs, or 19% of the total OTUs found in the study, were identified as being differentially abundant (Supplemental Table 7, First Tab). The following number of OTUs in dominant phyla (phyla in abundance > 1%) were affected: 2607 Ascomycetes, 669 OTUs from an unidentified fungal phyla, 291 Glomeromycota, 204 Basidiomycetes, and 41 Chytridiomycota.

When comparing plots treated with K to plots treated with N, 7406 OTUs (22% of total) exhibited differential abundance (Supplemental Table 7, Second Tab). The following number of OTUs in dominant phyla were affected: 2951 Ascomycetes, 794 OTUs from an unidentified fungal phylum, 322 Glomeromycota, 227 Basidiomycetes, and 42 Chytridiomycota.

When examining plots treated with K versus N+K treated plots, 7477 OTUs (22% of total population), exhibited differential abundance (Supplemental Table 7, Third Tab). The following number of OTUs in dominant phyla were affected: 3098 Ascomycete OTUs, 833 unidentified fungal phyla, 302 Glomeromycota, 233 Basidiomycetes, and 35 Chytridiomycota.

In general, fungi were present in higher abundances in K treated plots compared to N and N+K treated plots. For example, the Ascomycetes *Gaeumannomyces spp.* and *Magnaporthe spp.* were usually more abundant in K only treated plots, though a few individual OTUs exhibited highest abundance in plots treated with N+K. For example, two *Gaeumannomyces spp.* OTUs, OTU33852 and OTU6012, were most abundant in K treated plots (OTU counts- N = 11, K = 18, N+K = 1; N = 16, K = 20, N+K = 12, respectively. However, *Gaeumannomyces spp*. OTU26023 was most abundant in N+K treated plots (N = 1, K = 4, N+K = 39).

In general, Glomeromycetes identified as *Entrophosphora spp., Glomus spp., Rhizophagus intraradices,* and in the Paraglomerales, were present in all treatments, and always more abundant in K and N+K treated plots, and of lowest abundance in N treated plots. For example, *R. intraradices* OTU10133 was present in the following counts- N = 10, K = 23, N+K = 20, while *Entrophosphora spp.* OTU10153 was present in the following counts- N = 8, K= 10, N+K=14. Of course, occasional exceptions did exist. For example, OTU4544, representing *Archaeosporal spp.* (Glomeromycota), was present in only N+K treated plots (OTU count = 55), while *Entrophosphora sp*. OTU2448 was present in highest abundance in N only plots (N = 27, K = 23, N+K =4).

Field 2N Study: Microbial Diversity and Community Composition

Like the Field 1K study, rank abundance plots showed similar richness and evenness among samples for both archaea/bacteria and fungi (Figure 3A-D), and rarefaction analysis curves for all microbial groups reached a plateau (Figure 2A-D).

Alpha diversity calculated using the Shannon index is summarized in Table 2. Nonparametric two-way t-tests showed no significant difference in alpha diversity between the two treatments for either archaea/bacteria or fungi ($p = 0.327$; 0.501) (Table 2). As seen within the Field 1K study, archaea/bacteria diversity was higher than fungal diversity, regardless of treatment ($p = 0.05$).

In total, members of three archaea phyla and 47 bacterial phyla were identified from the Field 2N study. For archaea, total abundance averaged across all Field 2N samples was as follows: Crenarchaeota 3.5%, Parvarchaeota 0.1%, and Euryarchaeota 0.04%. For bacteria, total abundance averaged across all Field 2N samples was as follows: Proteobacteria 19.0%, Acidobacteria 17.2%, Chloroflexi 6.0%, OP11 3.3%,

Actinobacteria 2.9%, Planctomycetes 2.6%, Bacteroidetes 1.6%, TM7 1.5%, OD1 1.1%, and Verrucomicrobia 1.1%. The remaining 37 bacterial phyla were present in abundance <1%.

In total, six fungal phyla and one fungal-like phyla (Ichtyosporea) were identified from the Field 2N study. Total abundance averaged across all Field 2N samples was as follows: Ascomycota 34.6%, unidentified fungal genus 19.7%, and Basidiomycota 8.4%. The remaining phyla were present in abundance <1%. All taxa identified in the Field 2N study can be found in Supplemental Table 8.

Field 2N Study: Differential Abundance

Like the Field 1K Study, fertility treatments altered the abundance of archaea and bacteria communities, especially for Proteobacteria and Acidobacteria, the two most common bacterial groups identified in this study (Supplemental Table 9). The data presented below is summarized from Supplemental Table 9. When comparing plots treated with the low N rate to the higher rate of N, 13599 OTUs displayed differential abundance, representing just 10% of the total archaea/bacteria population (Supplemental Table 9, First Tab). The following number of OTUs in dominant phyla (phyla in abundance > 1%) were affected: 3713 in Proteobacteria, 2810 in Acidobacteria, 926 in Chloroflexi, 529 in Actinobacteria, 468 in the candidate bacterial division OP11, 379 in Planctomycetes, 294 in Bacteroidetes, 257 in candidate division TM7, 193 in Crenarchaeota, 143 in Verrumicrobia, and 125 in Gemmatimonadetes.

In general, archaea/bacteria were more abundant in plots receiving the low rate of N compared to the high rate of N. More specifically, counts of the Crenarchaeota clone

SAGMA-X, Acidobacteria, Alphaproteobacteria, and Xanthomonadaceae were always higher in plots receiving low N.

For fungi, 7226 OTUs exhibited differential abundance when comparing between low N and high N treatments, representing 31% of the total fungal population (Supplemental Table 9, Second Tab). Similar to the Field 1K study, this was particularly true for the Ascomycetes. The following number of OTUs in dominant phyla (phyla in abundance > 1%) were affected: 2695 Ascomycetes, 1143 from the unidentified fungal phyla, 307 Basidiomycetes, 166 Glomeromycetes, and 18 Chytridiomycetes.

In general, mycorrhizal fungi were more abundant in plots treated with the lower rate of N. For example, Glomeromycetes in *Entrophosphora sp., Glomus sp.,* and Paraglomerales were always more abundant in plots receiving low N. However, the Ascomycetes *Gaeumannomyces spp.* and *Magnaporthe spp.* were higher in plots receiving high N. For example, *Magnaporthe spp*. OTU631, was recovered 39 times in high N treated plots and only four times in low N treated plots. Similarly, *Gaeumannomyces spp.* OTU21797 was recovered 99 times in high N treated plots, and only five times in low N treated plots.

Turfgrass Pathogen Distribution

Eight turfgrass pathogens were identified in soil samples to the genus or species level using the custom designed turfgrass pathogen database (Supplemental Table 10), representing only 0.0306% of the total fungal organisms identified in both the field 1K and field 2N studies. Relative to all other turfgrass pathogens, the fungus *Microdochium nivale*, the causal agent of pink snow mold, and the fungus *Sclerotinia homoeocarpa*, the causal agent of dollar spot disease, both foliar pathogens, were present in the highest

abundance in all soil samples, at levels of 5.9 X 10^{-3} and 3.4 x 10^{-3}, respectively. *Laetisaria fuciformis*, the causal agent of red thread disease, and *Puccinia sp.,* the causal agents of rust diseases, also foliar pathogens, were present at the lowest levels (3.3 x 10^{-6} and 1.5 x 10^{-6}, respectively). *Colletotrichum cereale*, the incitant of the foliar and stem rot disease anthracnose, was only identified in two soil samples and at very low levels (2.2 x $10^{-5}$and 1.5 x 10^{-6}). The root infecting pathogens *Gaeumannomyces graminis*, the causal agent of take-all, and *Magnaporthiopsis poae*, the causal agent of summer patch were also identified, but at very low levels (2.19 x 10^{-5} and 2.31 x 10^{-6}, respectively). Plants did not display any symptoms of root infecting or foliar pathogens, except *C. cereale*.

DISCUSSION

The primary objective of this work was to determine if different levels of nitrogen and potassium inputs influence microbial diversity and/or alters community structure and distribution on an annual bluegrass putting green. When testing for presence/absence of OTUs between the fertility treatments, simulated power analyses, a tool commonly used by statisticians to estimate sample size (Campbell et al. 1995), indicated that additional sampling beyond 12 replications was necessary to detect significant differences, a valuable finding for designing future studies with similar scope. In the current study, power analyses were useful for evaluating data sets to identify potential differences between treatments; however this data cannot be used to draw precise conclusions about presence/absence of the microbial community, since these analyses are extrapolations and assume OTU counts would not change as sample size increases. Environmental sequence data can display extensive variation (O'Brien et al. 2005), thus OTU counts will likely change as the sample size. Therefore, the true utility of the power analyses is to demonstrate what may be accomplished with more samples. Regardless, the rarefaction curves presented here, the most common method used for assessing sufficient sampling (Wooley et al. 2012), showed that diversity reached saturation with the sampling method employed in this study. In addition, the rank abundance plots showed that the most abundant and dominant species were present at similar levels, indicating that the data collected in this study was a representative sampling of the dominant community in the soil of the annual bluegrass putting greens.

Consistent with the observations made in chapter 2, the soil associated with annual bluegrass putting green turf in this study displayed high microbial diversity for all

fertility treatments. In fact, the diversity metrics observed in this system are comparable to those observed from the soil of a range of biomes not receiving any fertility inputs (e.g., the polar desert, a hot desert, the arctic tundra, temperate grasslands, and tropical, temperate deciduous and coniferous, and boreal forests) (Fierer et al. 2012b). Golf courses are known to support biodiversity by providing vital habitats for aboveground flora and fauna (Jodice and Humphrey 1992, Terman 1997, Colding and Folke 2009), and the results described here suggest a similar response belowground for annual bluegrass putting greens. However, it is important to note that the Field 1K and Field 2N studies only had one sampling date, while chapter 2 reflects microbial communities from five samples conducted over 12 months. Very different proportions of total sequences were identified for archaea, bacteria, and fungi between the two chapters, a finding that could be directly related to the frequency of sampling. To accurately determine whether microbial diversity changes over time in such a turfgrass system, future research would need to be conducted as multiple samplings over an extended period (years).

The results presented here clearly show that archaea, bacteria and fungal abundance in annual bluegrass turf is changed as a result of nitrogen and potassium applications, a finding that is consistent with other studies (Elliot et al. 2003, Mueller et al. 2006, Wallenstein et al. 2006, Fierer et al. 2012a). However, changes were only observed in a relatively small percentage of the microbial community in both studies ($\leq$10% for archaea/bacteria; $\leq$31% for fungi). Likewise, while the abundance of species changed as a result of fertility treatments, overall diversity was maintained. A similar result was found when examining bacterial populations across nitrogen gradients in an established grassland and agricultural field, suggesting that fertility treatments shift

microbial communities to those capable of functioning in such environments (Fierer et al. 2012a). For example, higher N rates have been suggested to favor copiotrophs (ex. Alphaproteobacteria) than oligotrophs (ex. Acidobacteria) (Fierer et al. 2012). Copiotrophs are defined as organisms present in high nutrient environments, whereas oligotrophs are bacteria typically found in areas of low nutrient concentration (Koch 2001). The distribution of the latter group is thought to affect nutrient cycling and may be indicative of unhealthy soils, since low levels of oligotrophs have been associated with increased plant disease (Borrero et al. 2004, Kotsou et al. 2004). In fact, Acidobacteria, the second most abundant bacterium in both studies (~17% of total abundance), and was found in greater abundance in low N plots that were exhibiting severe foliar symptoms of anthracnose disease (data not shown). Following this logic, a higher abundance of Alphaproteobacteria would have been expected in plots receiving the highest N rates, 132 kg N $ha^{-1}yr^{-1}$ for the Field 1K study and 200 kg N $ha^{-1}yr^{-1}$ for the Field 2N study. However, Alphaproteobacteria, although a small portion (less than 1%) of the microbial community in these studies, were most abundant in low N treatments. Several theories could explain this result. Copiotrophic bacteria grow intensely after nutrients are applied, and their populations can quickly drop as nutrients are utilized (Niewiadomska 2015). Thus, it is possible changes in their population were not captured with the 11 September 2013 sampling, as fertility treatments were applied seven days earlier at a very low rate (4.9 kg N ha^{-1}), or, that higher application rates are required to increase the abundance of copiotrophs (see Fierer et al. 2012, Leff et al. 2015, Niewiadomska 2015). The quantity of N applied during the growing season in the aforementioned studies had a wider range of N applications (0.0 kg ha^{-1} yr^{-1} to 291 kg ha^{-1} yr^{-1}) then employed in the current

studies. Thus it is possible that the microbial community in the soil of annual bluegrass turf may respond differently given a more robust range of N applications. However, the seasonal rates employed in the Field 1K study (132kg ha^{-1} yr^{-1}) and the Field 2N study (100 kg ha-1 yr-1 and 200 kg ha^{-1} yr^{-1}) reflect annual quantities of N commonly used in the turfgrass industry based on best management practices for promoting healthy, disease-free annual bluegrass putting greens (Inguagiato et al. 2008). Thus, examining the impact of a wider range of N rates may result in increased microbial populations in the soil of annual bluegrass, but may not reflect best management practices.

Several interesting organisms were present in differential abundance in individual fertility treatments. In particular, the archaea clone SAGMA-X, was routinely identified in all samples (this chapter and chapter 2) and on all sampling dates. This organism was found in highest abundance in K and N+K treated plots in the Field 1K study. In chapter 2, it is hypothesized that this archaeon may be universal in distribution due to its ability to metabolize urea and oxidize ammonia. Thus, one would have expected to see higher abundance of SAGMA-X in the N only or N+K treatments in the Field 1K study, not the K only treatment, which only received an N application early in the season. Interestingly, archaea are known to rapidly uptake K^+ ions under high salt conditions to prevent water loss (Becker et al. 2014), evidence showing they can survive in K rich environments. Potassium was applied as KCl in this study, thus it is possible that the increase in Cl^- ions stimulated K^+ uptake to tolerate the temporary salt concentrations, allowing SAGMA-X to continue surviving. Regardless, the results indicate that the distribution and abundance of SAGMA-X is influenced by a variety of factors, and gene expressions studies will

need to be conducted under these varying conditions to determine exactly how K and N treatments affect ammonia oxidation in this system.

As in chapter 2, a large number of mycorrhizae were observed in this study. In other agricultural settings, fertilizer and pesticide applications have been associated with reductions of mycorrhizae (Sattelmacher et al. 1991). Creeping bentgrass (*Agrostis stolonifera*) putting greens have been shown to support more mycorrhizae than neighboring turf areas receiving lower inputs, suggesting that these fungi can thrive in such a highly maintained system (Koske et al. 1997). The abundance of Glomeromycota identified in this study, which received extensive pesticide inputs, supports this theory. However, different levels of mycorrhizae were observed depending on fertility treatment, suggesting that not all mycorrhizae respond similarly. In general, the abundance of fungi such as *Glomus spp., Entrophosphora sp.,* and *R. intraradices* were highest in the K and N+K (Field 1K) treatments. If N alone exerted the strongest influence on mycorrhizae, populations would be expected to be highest in the K only treatment. In a long-term fertilizer experiment, Wang et al. (2009) showed that different fertilizer applications stimulated various stages of growth in different mycorrhizal species. For example, *Glomus mosseae* produce more spores under N+P+K treatments applied as urea, calcium phosphate and potassium chloride, whereas spore density of *Scutellospora pellucida* was highest under just the N and K treatment (Wang et al. 2009). This finding could explain why some OTUs identified in the field 1K or field 2N study, such as *Archaeosporal spp.,* and certain *Entrophosphora spp.* were only found in certain treatments. The presence of multiple fungal species could explain why different OTUs of the same genus were most abundant in different fertility treatments. Nevertheless, the overall results show that

mycorrhizae populations can be altered via fertility applications, holding promise that these fungi can be externally manipulated and utilized to promote plant health. Moving forward, it would be helpful to use culture-based studies along with genetic analysis to identify the mycorrhizae present in annual bluegrass putting green turf and to facilitate controlled greenhouse experiments with these fungi to determine what benefit their presence may provide.

Turfgrass pathogens were present at very low levels throughout the Field 1K and Field 2N study, and level of abundance was similar to that observed in chapter 2. In fact, all were reported at levels between 10^{-3} to 10^{-6}. These numbers do not give a good indication of the number of the potential disease-causing propagules present; however, they can be used to estimate such factors. For example, a real-time PCR assay designed to detect rust fungi could detect as few as 50 urediniospores, representing a DNA concentration of 1 pg (10^{-12}) (Beirn et al. 2011). Rust fungi were detected in the current study at 10^{-6}, suggesting that approximately 1 urediniospore is present in 1.2 µg of soil. This is not surprising, since rust, and the majority of pathogens identified to the species level, were primarily foliar and crown-infecting pathogens and thus would not be expected to occur in the soil in high concentrations. The potential soil-borne pathogens *Gaeumannomyces spp.* and *Magnaporthe spp*. were also identified at low levels, but a species-rank could not be determined for these fungi, preventing their positive identification as a turf pathogen. Unlike chapter 2, the antagonistic bacterium *Brevibacterium* was not identified, but *Burkholderia* and *Pseudomonas* were present. There was no differential abundance observed for *Burkholderia* or *Pseudomonas* across any fertility treatments. This finding further supporting the theory developed in chapter 2

that the stability and widespread nature of these bacteria, combined with their anti-fungal properties, deserve further investigation as potential biocontrol agents.

In addition to *Burkholderia* and *Pseudomonas*, a large number of Xanthomonads were observed in this study, and they were most abundant in K and low N treatments. Interestingly, *Gaeumannomyces spp.* and *Magnaporthe spp.* (genera containing several important root-infecting pathogens of turf) were also most abundant in these two treatments. *Stenotrophomonas maltophilia*, a Xanthomonad, has been successfully used as a biocontrol agent for summer patch disease, where the disease was reduced by more than 70% in Kentucky bluegrass (Kobayashi et al. 1995). Thus it is possible that the abundance of Xanthomonads may be directly related to the low level of turf pathogens, a potential food source.

While a small subset of OTUs exhibited differential abundance when exposed to different fertility treatments, others did not. For the microorganisms that were unaffected by fertility treatments, their ability to tolerate a wide range of nitrogen and potassium rates is also quite interesting. For example, the fungal genus *Pochonia spp.* was identified in the Field 1K study, and its abundance did not change across nitrogen and potassium treatments. *Pochonia* is best known as a parasite of root nematodes, and several strains within this genus have been utilized as successful nematicides around the world (Manzanilla-Lopez et al. 2013). While the benefit, if any, that *Pochonia* may provide for putting green turf, is not yet understood, its activity against nematodes suggests that it too may hold promise as a potential biocontrol agent. The experimental design, DNA, and statistical methods developed here should serve as a foundation for developing future metagenomics-based studies of the microbial community of annual bluegrass turf and

other golf course ecosystems, thus encouraging additional investigations into potentially beneficial organisms like *Pochonia spp*..

Interestingly, few OTUs were present in abundances greater than 1% in all samples in both the Field 1K study (seven OTUs) and the Field 2N study (10 OTUs). Yet, thousands of OTUs could be identified taxonomically. Traditionally, taxa present in low abundance are filtered from downstream analyses in favor of taxa that dominate the sample (Sogin et al. 2006). Only recently have taxa present in lower abundance, often termed 'rare taxa' or the 'rare microbiome', begun to be explored as important contributors to ecosystems (Sogin et al. 2006). The vast number of OTUs present in abundance less than 1% in the current two studies, suggest that the soil of annual bluegrass putting green turf possesses a rare microbiome waiting to be explored.

The different microbiota displayed across the three studies (chapter 2 and 3) raises interesting questions about how other factors, in addition to sampling, may be influencing soil microbial communities in annual bluegrass putting greens. For example, the two studies sampled here were established on the same soil type and are in relatively close proximity to one another in the same field (52 m). However, the Field 1K study was established approximately seven years before the Field 2N study. Microbial biomass has been shown to accumulate as putting greens age (Kerek et al. 2002), and microbial communities in older turfgrass stands are known to diverge from younger stands based on cluster analyses (Yao et al. 2006). Thus, turf age could be a major contributing factor to the different populations of microorganisms observed in the two study sites sampled here. However, the study site sampled in chapter 2 was established in the same year as the Field 2N study, but was a border area adjacent to this study and received only moderate

fertility and maintenance. Thus, this may explain the lack of consistency between the microbial communities described in chapter 2 with those observed in the Field 2N study.

The Field 1K study and the Field 2N study sampled here, and the study site sampled in chapter 2, were maintained by different individuals. As such, each employed slightly different management regimes that could also have contributed to variation observed among microbiota. For example, mowing regimes differed slightly in the Field 1K study and the Field 2N study, (see methods section), raising questions about the impact of common management practices utilized on annual bluegrass putting green turf and how they may be altering the soil microbial community. Mowing has been shown to impact the abundance of ammonia-oxidizing microorganisms in a grassland ecosystem composed of multiple plant species, but the height of cut examined in their study was 10 cm, versus non-mown treatments (Chen et al. 2014). In the two studies described here, the height of cut differed by 0.4 mm (2.8 vs 3.2 mm). While this may seem insignificant, previous studies conducted at the current site have reported major differences in anthracnose disease severity when annual bluegrass turf was maintained at these two cutting heights (Inguagiato et al. 2008). Therefore, it is possible that the microbial community in the soil of these sites may also be affected by such small differences in cutting height which can affect the moisture and temperature of the soil, factors that could affect microbial populations. Although further research is needed to address this question, this finding highlights the need to consider management practices (e.g., irrigation practices, topdressing rate and frequency, cultivation practices, etc.) employed on field sites when designing future metagenomics studies. And, just as important, whether we can use these routine management practices to potentially select for a

desirable soil microbial community to promote improved plant health and productivity? Considering these factors, metagenomic next-generation sequencing will be an important tool in the future to gain more insight into the functionality of the microbial groups identified in this study.

A substantial portion (39.5%) of the sample from the field 1K and the field 2N study could not be identified using the Greengenes or UNITE + INSDC databases. This is not uncommon, as soil metagenomics studies often reveal an array of microorganisms that have previously not been identified using conventional methods (Riesenfeld et al. 2004, Kent and Triplett 2006). For example, in the soil in both an oak and a mixed-stand forest, unclassified bacteria and fungi represented 20% and 11% of the samples, respectively (Buee et al. 2009, Uroz et al. 2010). Similarly, unclassified bacteria represented 16% of the sugar beet rhizosphere (Mendes et al. 2011). When this data was reevaluated with the updated Greengenes database a year later, the percentage of unclassified bacteria decreased by nearly 50% (Mendes et al. 2013). In this analysis, the reference sequence database and that used in chapter 2 represent different versions of the same database, and likely account for some of the fundamental differences in the sequence data between the two studies. For example, in this study, plant DNA accounted for 0.09% of the total sequences in this study, while in chapter 2, plant DNA was only present as 2.65×10^{-5} of the total sequence data. Because sampling and PCR methods were the same, this is likely due to slight differences in the reference database with regards to plant DNA. As reference databases continue to expand and more soil microbial taxa are described, one would expect to see similar improvements in the percentage of overall microorganisms identified. However, unless custom, ecosystem-specific

reference databases are also integrated into metagenomics analyses, organisms of interest may still be missed by existing databases. For example, none of the 41 fungal and oomycete pathogens included in our custom turf database were present at the taxonomic level of species in the UNITE + INSDC databases. While many were detected at relatively low levels, these organisms would not have been detected at all using existing resources, and information on an important component of the turfgrass rhizosphere would be missed. Until custom databases are developed and implemented, the function and role of many other organisms of interest will remain a mystery.

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Table 1. Results of simulated power analyses demonstrating the effect of sample number on presence/absence of archaea/bacteria and fungi between fertility treatments, measured as the numbers of OTUs, in Field Studies 1K and 2N

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		Field 1K Study			Field 2N Study
	Number of Samples Per Treatment[a]	**Significant OTUs[b]**			
		K[c] vs.N[d]	**N+K[e] vs. N**	**K vs. N+K**	**Low N[f] vs. High N[g]**
Archaea/Bacteria	12[h]	none	none	none	none
	20	182	140	128	135
	30	3829	3973	15425	5437
Fungi	12[h]	none	none	none	none
	20	194	144	179	160
	30	3227	1171	6458	3055

[a]Simulated power analyses were completed by maintaining the proportion of observed sample counts in the 2 x 2 table, and increasing the sample size (n) for each treatment by 2. Values of n examined ranged from 14 (the original sample size of 12 + 2) to 50. Once sample size was artificially increased, a 2 x 2 Fisher's exact test was performed on the simulated data. Only simulated sample sizes of 20 and 30 are included below.
[b]OTUs were considered significant if p-values adjusted using the Benjamini-Hochberg FDR were <0.05.
[c]K was applied every 14 days as KCl at a rate of 13.7 kg ha^{-1} from 23 April to 8 November 2013.
[d]N was applied as urea every 14 days at a rate of 4.9 kg ha^{-1} from 23 April to 8 November 2013.
[e]N+K was applied every 14 days as urea and KCl as a combined treatment (1:1, N+K molar-adjusted ratio) from 23 April to 8 November 2013.
[f]Low rate of N was 4.9 kg N ha^{-1} of urea every 14 days from 8 April to 30 September 2013.
[g]High rate of N was 4.9 kg N ha^{-1} of urea every 7 days from 8 April to 30 September 2013.
[h]Twelve, the actual number of samples collected in this study. Note, presence/absence of OTUs .

Table 2. Pairwise comparisons of alpha diversity indices for archaea/bacteria and fungi as affected by fertility treatment using nonparametric two-way t-tests.

Field 1K Study	***Archaea/Bacteria*[a]**							
	Factor 1	**Factor 2**	**Factor 1 Shannon Index Mean[c]**	**Factor 1 Standard Deviation**	**Factor 2 Shannon Index Mean**	**Factor 2 Standard Deviation**	**t stat**	**p-value**
	K only[d]	N+K[e]	9.207	0.307	9.012	0.434	1.222	0.774
	K only	N only[f]	9.207	0.307	9.002	0.393	1.365	0.549
	N+K	N only	9.012	0.434	9.002	0.393	0.052	1.000
	Fungi							
	Factor 1	**Factor 2**	**Factor 1 Shannon Index Mean**	**Factor 1 Standard Deviation**	**Factor 2 Shannon Index Mean**	**Factor 2 Standard Deviation**	**t stat**	**p-value**
	K only	N+K	7.019	0.658	7.167	0.444	-0.618	1.000
	K only	N only	7.019	0.658	7.103	0.468	-0.344	1.000
	N+K	N only	7.167	0.444	7.103	0.468	0.330	1.000
Field 2N Study	***Archaea/Bacteria*[b]**							
	Factor 1	**Factor 2**	**Factor 1 Shannon Index Mean**	**Factor 1 Standard Deviation**	**Factor 2 Shannon Index Mean**	**Factor 2 Standard Deviation**	**t stat**	**p-value**
	Low N[g]	High N[h]	8.925	0.481	9.097	0.318	-0.988	0.327
	Fungi							
	Factor 1	**Factor 2**	**Factor 1 Shannon Index Mean**	**Factor 1 Standard Deviation**	**Factor 2 Shannon Index Mean**	**Factor 2 Standard Deviation**	**t stat**	**p-value**
	Low N	High N	7.025	0.324	7.185	0.689	-0.699	0.501

[a]Data from a depth of 1000 seqs/sample.
[b]Data from a depth of 4000 seqs/sample.
[c]Shannon Index as log base 2 using output from QIIME.
[d]K was applied every 14 days as KCl at a rate of 13.7 kg ha^{-1} from 23 April to 8 November 2013.
[e]N+K was applied every 14 days as urea and KCl as a combined treatment (1:1, N+K molar-adjusted ratio) from 23 April to 8 November 2013.
[f]N was applied as urea every 14 days at a rate of 4.9 kg ha^{-1} from 23 April to 8 November 2013.
[g]Low rate of N was 4.9 kg N ha^{-1} of urea every 14 days from 8 April to 30 September 2013.
[h]High rate of N was 4.9 kg N ha^{-1} of urea every 7 days from 8 April to 30 September 2013.

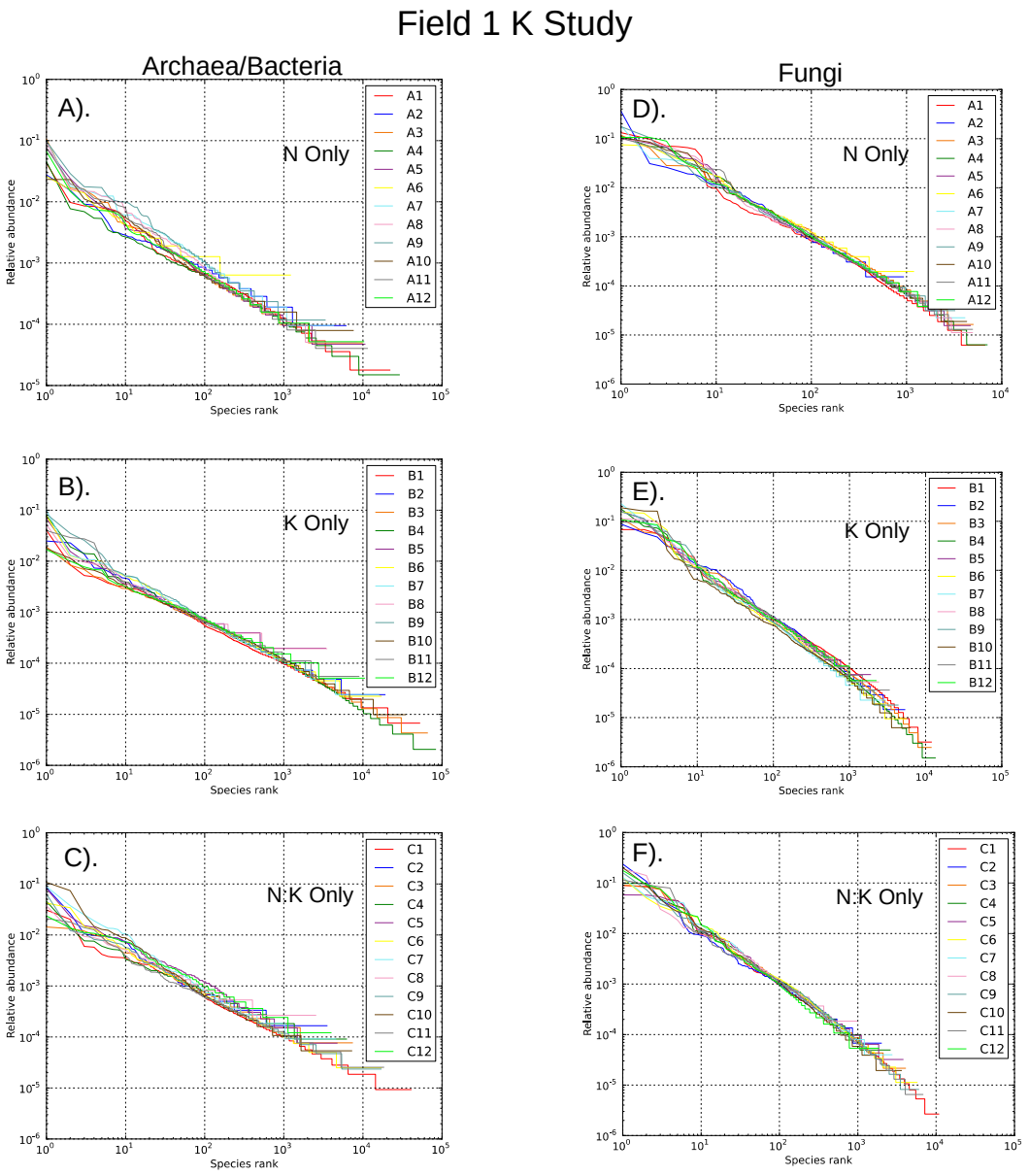

Figure 1. Rank abundance plots for the Field 1K study. Species are plotted on the x-axis and relative abundance are plotted on the y-axis. Species rarity increases moving right along the x-axis. Twelve samples were taken from each fertility treatment; each sample is represented by a colored line. Fertility treatments were as follows- K was applied every 14 days as KCl at a rate of 13.7 kg ha^{-1} from 23 April to 8 November 2013, N was applied as urea every 14 days at a rate of 4.9 kg ha^{-1} from 23 April to 8 November 2013, and N+K was applied every 14 days as urea and KCl as a combined treatment (1:1, N+K molar-adjusted ratio) from 23 April to 8 November 2013. In general, dominant species (left on the x-axis) exhibit similar abundances within their respective fertility treatments. A-C). Archaea/bacteria, D-F). Fungi.

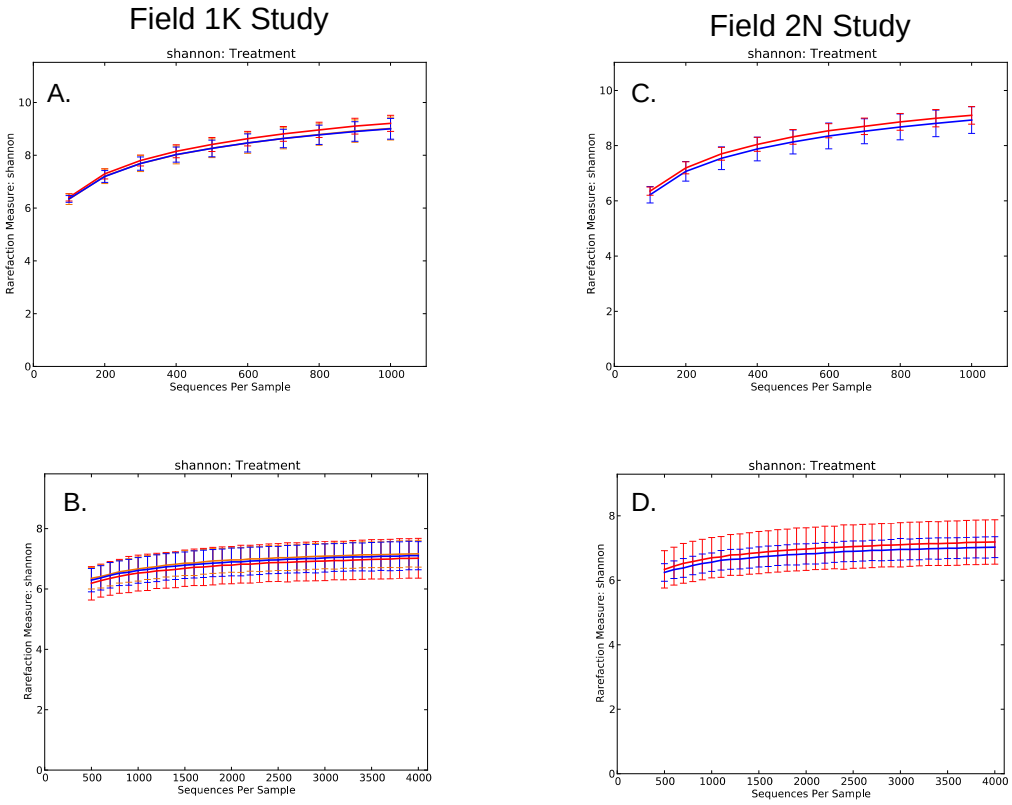

Figure 2. Diversity rarefaction curves, where alpha diversity is plotted on the y-axis and number of sequences is plotted on the x-axis. As sequences per sample increase, microbial diversity plateaus (reaches saturation). Fertility treatments for the Field 1K study were as follows- K was applied every 14 days as KCl at a rate of 13.7 kg ha^{-1} from 23 April to 8 November 2013, N was applied as urea every 14 days at a rate of 4.9 kg ha^{-1} from 23 April to 8 November 2013, and N+K was applied every 14 days as urea and KCl as a combined treatment (1:1, N+K molar-adjusted ratio) from 23 April to 8 November 2013. For the Field 2N study, the low rate of N was 4.9 kg N ha^{-1} of urea every 14 days from 8 April to 30 September 2013 and the high rate of N was 4.9 kg N ha^{-1} of urea every 7 days from 8 April to 30 September 2013. A). Archaea/bacteria for the Field 1K study, where red = N only, blue = K only, and orange = N+K. Note, orange line is overlapped by blue line. B). Fungi for the Field 1K study, where red = N only, blue = K only, and orange = N+K. C). Archaea/bacteria for the Field 2N study, where red =low N, blue = high N. D). Fungi for the Field 2N study, where red =low N, blue = high N.

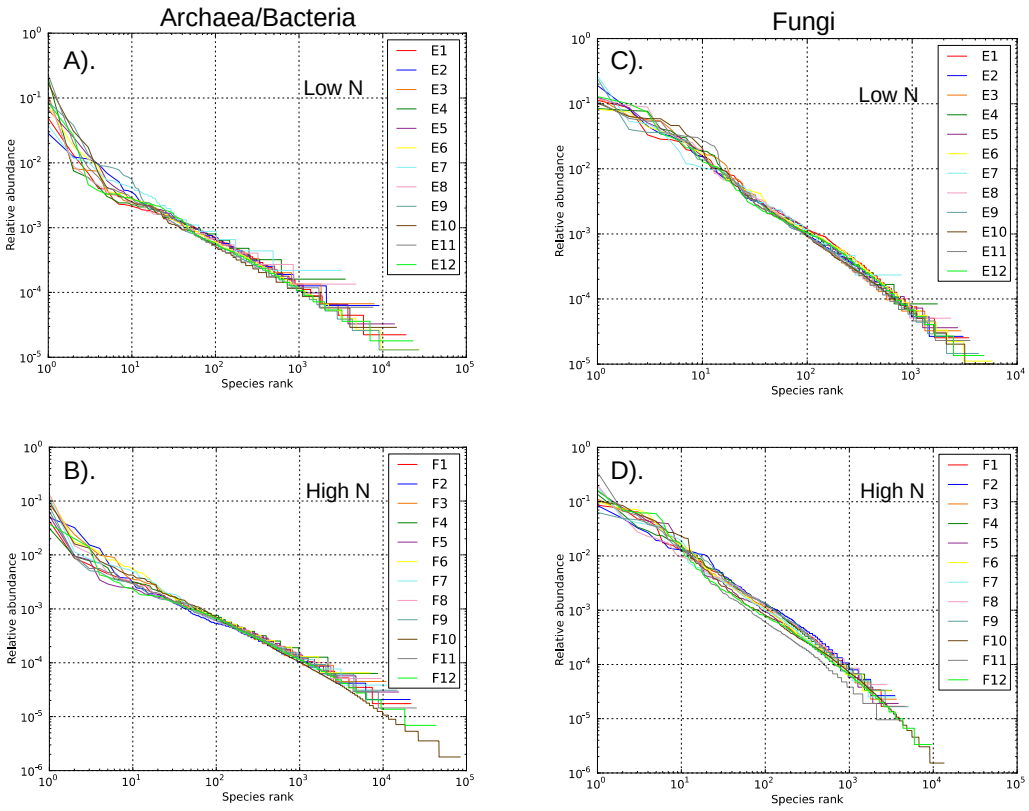

Figure 3. Rank abundance plots for the Field 2N study. Species are plotted on the x-axis and relative abundance are plotted on the y-axis. Species rarity increases moving right along the x-axis. Twelve samples were taken from each fertility treatment; each sample is represented by a colored line. Fertility treatments were as follows- the low rate of N was 4.9 kg N ha^{-1} of urea every 14 days from 8 April to 30 September 2013 and the high rate of N was 4.9 kg N ha^{-1} of urea every 7 days from 8 April to 30 September 2013. In general, dominant species (left on the x-axis) exhibit similar abundances within their respective fertility treatments. A-C). Archaea/bacteria, D-F). Fungi.

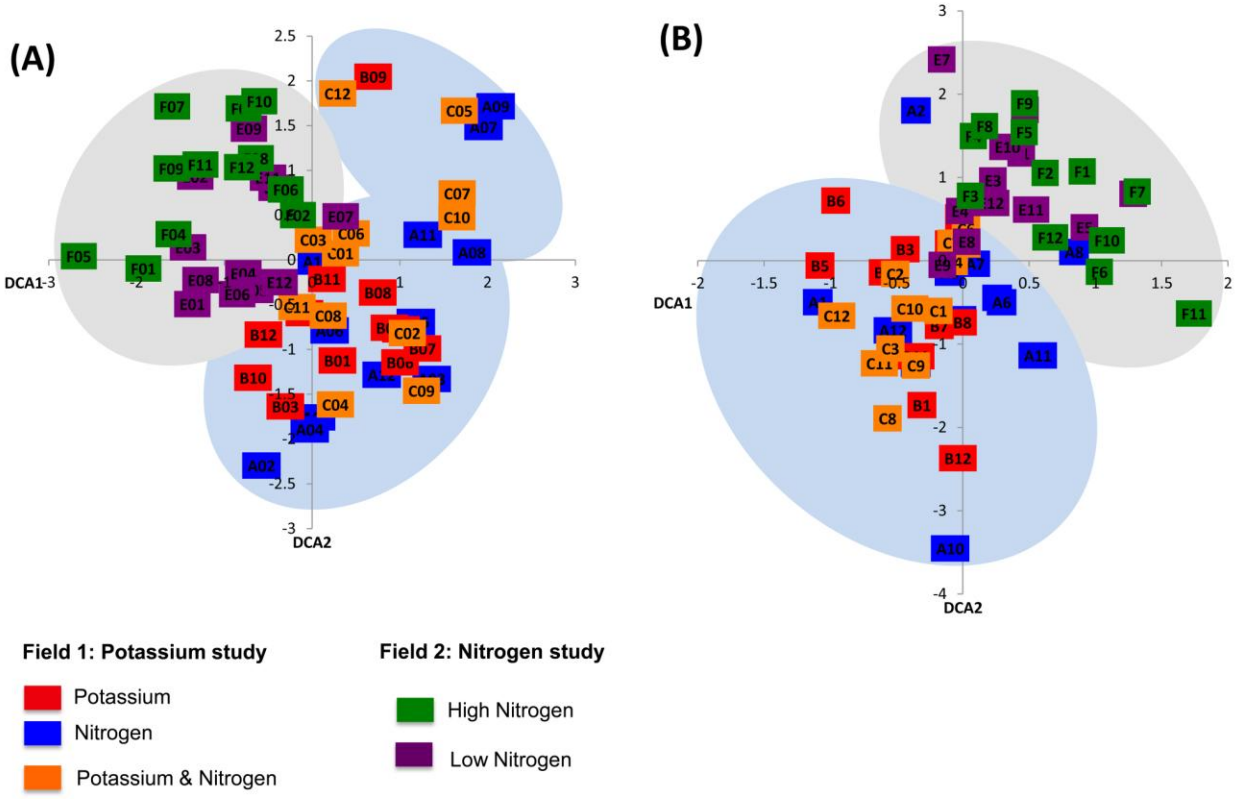

Figure 4. Multivariate detrended correspondence analysis of microbial across 60 sample sites. Analyses are based on genus level OTU assignments. Fertility treatments for the Field 1K study were as follows- K was applied every 14 days as KCl at a rate of 13.7 kg ha^{-1} from 23 April to 8 November 2013, N was applied as urea every 14 days at a rate of 4.9 kg ha^{-1} from 23 April to 8 November 2013, and N+K was applied every 14 days as urea and KCl as a combined treatment (1:1, N+K molar-adjusted ratio) from 23 April to 8 November 2013. For the Field 2N study, the low rate of N was 4.9 kg N ha^{-1} of urea every 14 days from 8 April to 30 September 2013 and the high rate of N was 4.9 kg N ha^{-1} of urea every 7 days from 8 April to 30 September 2013. (A) Archaeal and bacterial communities (B) Fungal communities

DISSERTATION SUMMARY

The overall goal of this dissertation was to advance our understanding of the microbial community present in the soil of annual bluegrass putting green turf. By employing state of the art molecular technologies, I was able to characterize resident microbial populations, including pathogens and benign species. As a result of this research, insights into the distribution and abundance of the resident microbial community in a turfgrass system have been developed. Across all three studies, widespread microbial diversity was found. Above ground, the variation within one pathogenic species, *Colletotrichum cereale*, was highlighted in the form of two distinct genetic lineages, termed clade A and B. The two lineages appear to have a host and geographic preference, with clade A predominating in the southern U.S., and both clades found in equal frequencies in the northern U.S. However, clade B was recovered more frequently on annual bluegrass than creeping bentgrass in northern regions, though both clades were capable of cohabitating together within annual bluegrass putting green turf. In the rhizosphere, the tremendous diversity of microbes inhabiting the environment spanned three kingdoms of life, encompassing over 50 phyla, and thousands of individual species. This inventory of microbes included many organisms that could possibly play important beneficial roles in golf course putting greens, such as antibiotic producers, potential biocontrol agents, mycorrhizal species, nematode parasites, and nitrogen fixers. Thus, despite a soil environment with high sand content and extensive management inputs, annual bluegrass putting green turf can and does support a vast microbial community. This was an especially surprising finding of the research, given the input-

intensive management practices, such as regular fertilizer and pesticide applications, and daily mowing employed in this system.

Of course, it remains to be determined what this microbial community means with respect to the health and function of this ecosystem. Moving forward, the key to harnessing these communities for promoting plant health and suppressing pathogens, will lie in not only identifying these organisms, but in understanding their function and how they are affected by management practices. I have demonstrated that below ground microbial communities are altered as a result of fertility treatments, but it is not yet clear whether the net impact is positive or negative. For example, the severity of anthracnose disease, caused by *C. cereale,* can be significantly reduced with the addition of specific fertilizer inputs, which is thought to be a byproduct of improved plant health. Is the presence or abundance of *C. cereale* also changed as a result of fertility application? Or is anthracnose disease reduced because beneficial soil microbial communities are stimulated, supporting healthier plants? Alternatively, are any of the potential beneficial archaea, bacteria and fungi described in this dissertation capable of naturally suppressing aggressive pathogens, such as *C. cereale*? Understanding the links between above and below ground microbial communities and their plant hosts will be necessary to advance phytobiome research in the turfgrass system. Conducting gene expression studies to determine which organisms are active in this system and how they may contribute to plant health will be essential to better understand these associations. Integrating next-generation sequence studies with more traditional approaches, such as culturing, should also be considered. For example, collecting isolates of mycorrhizae found in ABG putting green turf would allow controlled, greenhouse-based experiments to be conducted

to determine under what conditions, and the frequency, that these fungi colonize annual bluegrass and what benefit they may provide.

Importantly, as one of the first bodies of work describing microbial communities in turf using advanced molecular technologies, protocols developed here should help researchers to better examine the microbial community in the turfgrass ecosystem in the future. For example, the data presented in this dissertation highlights the care that must be taken when conducting such studies, particularly with respect to sample size (number of replications), DNA extractions, and data analysis. It is apparent from the current studies that extensive initial sampling is needed to define within site variation, in order to determine how many samples are needed to answer specific research questions of interest. Using simulated power analyses described here to evaluate variability in microbial communities and estimate sufficient samples parameters will be of great importance when addressing these questions. Of course, the results of such analyses must still be interpreted with caution. Taking additional samples may not always be feasible, and will lend itself to other potential problems, such as the large amounts of data produced when sequencing additional samples. Thus, it is likely that a compromise will need to be explored for each system - one where enough samples are collected to address questions of interest, but still represent a manageable number of samples to process and analyze.

In addition to considering sampling parameters for future studies, laboratory concerns must also be addressed. Common laboratory reagents do contain DNA contamination, and this may bias downstream analyses. Similarly, poor-yielding DNA extraction kits can be problematic, in that only a portion of the microbial community may

be captured. Therefore, ensuring sufficient yield from extractions must be considered before embarking on large scale studies. A great deal of care was taken when evaluating extraction kits and procedures in the current research to take into account resident microbes, and this should serve as a starting point for researchers who wish to conduct similar research from golf course putting greens.

Finally, the generation of large scale datasets presents a unique challenge, such as data storage and short comings associated with currently available analytical techniques. As a result, assessing sufficient data processing and storage capacity prior to conducting next-generation sequencing studies of turfgrass systems should not be overlooked. With these challenges in mind, it will be possible to conduct environmental marker studies and collect meaningful research. We are only at the beginning of phytobiome research, particularly with respect to the turfgrass ecosystem, and the field is likely to continue to expand as technologies become more sensitive and affordable. It is my hope that the methods and the protocols developed in this dissertation will continue to be applied to turfgrass systems, allowing novel areas to be explored and new questions to be addressed.