Accurate identification and grouping of *Rhizoctonia* isolates infecting turfgrasses in MD and VA and their sensitivity to selected fungicides *in vitro*

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ABSTRACT

Rhizoctonia blight (sensu lato) is a common and serious disease of many turfgrass species. The most widespread causal agent *R. solani* consists of several genetically different anastomosis groups (AGs) and subgroups. Though anastomosis or hyphal fusion reactions have been used to group *Rhizoctonia* species, they are time consuming and sometimes difficult to interpret. Anastomosis reactions are incapable of identifying isolates belonging to different AG subgroups within an AG. This study evaluated molecular techniques in comparison with traditional anastomosis grouping (AG) to identify and group isolates of *Rhizoctonia*. More than 400 *Rhizoctonia* isolates were collected from diseased turfgrass leaves from eight geographic areas in Virginia and Maryland. A random sample of 86 isolates was selected and initially characterized by colony morphology, nuclei staining and anastomosis grouping. Molecular identification was performed by analysis of rDNA-ITS region and DNA fingerprinting techniques universally primed PCR (UP-PCR) and amplified fragment length polymorphism (AFLP). The cladistic analysis of ITS sequences and UP-PCR fragments supported seven clusters. Isolates of *R. solani* AG 1-IB (n=18), AG 2-2IIIB (n=30) and AG 5 (n=1) clustered separately. *Waitea circinata* var. *zeae* (n=11), and var. *circinata* (n=4) grouped separately. A cluster of six isolates (UWC) did not fall into any known *Waitea* group. Most of the binucleate *Rhizoctonia*-like fungi (BNR) (n=16) grouped separately. AFLP grouping also largely agreed with the above results. However, UWC isolates clustered into two groups. Molecular analyses corresponded well with traditional anastomosis grouping by clustering isolates within an AG or AG subgroup together. UP-PCR cross-hybridization could distinguish closely related *Rhizoctonia* isolates to their infraspecies level. Genetically related isolates belonging to the same AG subgroups cross-hybridized strongly, while isolates of different AGs did not cross-hybridize or did so weakly. Sequence-characterized amplified region (SCAR) markers were generated from UP-PCR products to

identify isolates of major pathogenic groups AG 1-IB and AG 2-2IIIB. Specific primer pairs successfully distinguished isolates of AG 1-IB and AG 2-2IIIB from isolates of other AGs. Sensitivity of *Rhizoctonia* species and AGs was tested *in vitro* to commercial formulations of iprodione, triticonazole and pyraclostrobin. *W. circinata* isolates were moderately sensitive to iprodione while isolates of *R. solani* and BNR were extremely sensitive. Isolates of AG 2-2IIIB showed less sensitivity to triticonazole than other *Rhizoctonia* isolates. *W. circinata* var. *zeae* isolates were moderately sensitive to pyraclostrobin while most of the other isolates were extremely sensitive.

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ATTRIBUTION

Several colleagues assisted with the writing and guidance of the research work presented in Chapters 2, 3 and 4 of this dissertation. The following is a brief description of their contributions.

Dilip Lakshman, Floral and Nursery Plants Research Unit, Beltsville Agricultural Research Center-West, Beltsville, MD, 20705
Dr. Lakshman provided majority of the funding for the research and also expert advice and guidance throughout the research work. He was the principal investigator for the grant provided by United States Golf Association (USGA) which supported this research and listed as a co-author in Chapters 2, 3 and 4.

Scott Warnke, Floral and Nursery Plants Research Unit, Beltsville Agricultural Research Center-West, Beltsville, MD, 20705
Dr. Warnke helped in securing the grant offered by USGA, which supported the majority of the expenses of the research work. He is listed as a co-author of the Chapter 2.

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Dr. Horvath served as the chair while he was a faculty member of Virginia Tech and once moved to University of Tennessee, he served as a co-chair. He provided partial funding and guidance during the implementation and completion of the research project and dissertation. He is listed as co-author of publications represented in Chapters 2, 3, and 4.

TABLE OF CONTENTS

LIST OF FIGURES

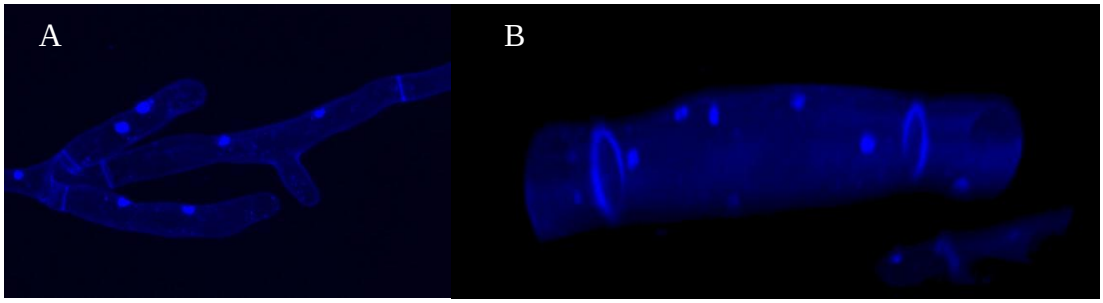
LIST OF TABLES

CHAPTER 1

Introduction

Rhizoctonia blight (sensu lato), which is a common and serious disease of many turfgrass species, has a wide distribution in warm humid and warm tropical climatic zones (7). In the southern United States, *Rhizoctonia* blight poses a major threat to successful growth and maintenance of tall fescue (*Festuca arundinacea* Schreb.), St. Augustinegrass [*Stenotaphrum secundatum* (Walter) Kuntze], zoysiagrasses (*Zoysia* spp.) and creeping bentgrass (*Agrostis stolonifera* L.) (7). Furthermore, isolates of *Rhizoctonia* have been identified from several other cool season and warm season grasses such as Kentucky bluegrass (*Poa pratensis* L.), perennial ryegrass (*Lolium perenne* L.) and bermudagrass [*Cynodon dactylon* L. (Pers.)] (19; 34; 55). In the early twentieth century, scientists thought *R. solani* was the only species within the genus to cause disease in turfgrasses. F.W Taylor, whose lawn served as the original material for disease diagnosis, named the disease "brown patch" (7). However, studies in the 1980's led to identification of additional *Rhizoctonia* species. These species have been assigned to two groups, viz. multinucleate or binucleate, based on the number of nuclei present in vegetative hyphal cells (Figure 1.1) (7). *Rhizoctonia solani* Kühn, *R. oryzae* Ryker and Gooch and *R. zeae* Voorhees have more than two nuclei per cell. Binucleate *Rhizoctonia* consists of more than 40 taxa (7). Several of them have teleomorphs in the genera *Ceratobasidium* D.P. Rogers and *Tulasnella* J. Schröt. Among binucleate species, *R. cerealis* Van der Hoeven is the most important species, causing yellow patch of turfgrasses (22).

Figure 1.1 Binucleate *Rhizoctonia cerealis* (A) and multinucleate *R. solani* (B) observed under a confocal microscope after staining with 4´, 6-diamidino-2-phenylindole (DAPI).



Microscopically, in the asexual state all *Rhizoctonia* species look more or less similar, i.e. nonsporulating mycelia with 90 degree branches having dolipore septa (Figure 1.2). Hyphal branching usually takes place close to septa. Initially, branches look acute angular but with maturity they become approximately perpendicular to the parent hypha. Hyphae have a distinct constriction at branch points (42). But when spores are induced or detected on rare occasions they can look quite different and thus have a second, sexual name or the teleomorphic form (53). The teleomorphic genera of *Rhizoctonia* species fall into the Ceratobasideaceae family in Tulasnellales order of the Basidiomycotina subdivision (7). Some *Rhizoctonia* species can be distinguished by colony morphology on potato dextrose agar (PDA). For instance *R. solani* forms a buff to brown mycelial mat on PDA whereas *R. oryzae* and *R. zeae* form a white to buff to salmon colored colony (7). The color of mature sclerotia also differs for these species. Sclerotia are formed by compaction of specialized hyphae called monilioid cells (Figure 1.3) and are capable of overwintering. However, in *Rhizoctonia* species, sclerotia do not differentiate into rind and medulla (42). Therefore some scientists prefer to call them bulbils. *R. solani* produces brown to dark brown sclerotia on the agar surface. The size, shape and abundance of sclerotia vary greatly for *R. solani* depending on the anastomosis group of the isolate. Sclerotia of *R. oryzae* are irregular in shape, salmon color and form on the surface as well as submerged in the medium. On the other hand *R. zeae* has orange colored, more or less uniformly spherical and frequently submerged sclerotia. Even with this macroscopic distinction in culture, some isolates of *Rhizoctonia* may often require additional morphological and physiological characteristics for identification (7).

Figure 1.2 Microscopic view of a *Rhizoctonia* hypha.

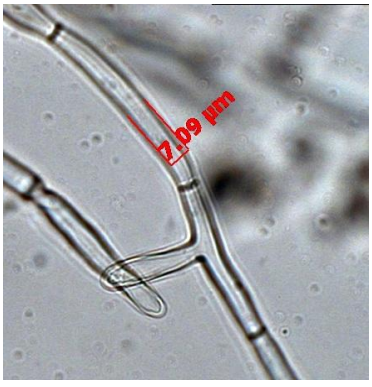
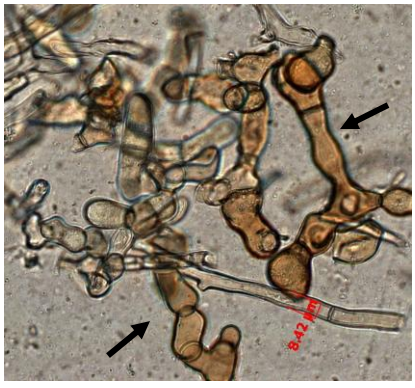


Figure 1.3 Monilioid cells of *Rhizoctonia solani*.



The latest findings describe five diseases caused by *Rhizoctonia* species on turfgrasses. Of these, brown patch caused by *R. solani* (teleomorph: *Thanatephorus cucumeris* (Frank) Donk) is the most common disease (42). Large patch, predominantly a disease of warm season turfgrass, is also caused by *R. solani* but belongs to a different anastomosis group viz. AG 2-2LP. Though these two diseases favors warm humid weather (>25°C), the time of attack depends on the grass type. Mostly, cool season grasses (e.g. creeping bentgrass, tall fescue and Kentucky bluegrass) are infected during summer whereas warm season grasses (St Augustinegrass, bermudagrass and zoysiagrass) are susceptible in the spring and fall when grasses are either breaking dormancy or approaching dormancy, respectively (7). The ability of *R. solani* isolates to cause disease under different climatic conditions indicates presence of subpopulations within the species. Causal agents of *Rhizoctonia* blight can over-winter as sclerotia or as mycelia in the plant debris. They can also survive as saprophytes when conditions are not favorable (48). Studies done on *R. solani* revealed that when the temperature rises above 16°C, the sclerotia begin to germinate and the fungus spreads in a circular manner. The pathogen begins to parasitize the grass under high humidity and day/night temperatures are at least 30°C/20°C. If the temperature is low and the plant is healthy, *R. solani* grows as a saprophyte and causes minimal infection to grasses (48). The main point of entry for the pathogen is the cut leaf tip. However, the fungus can also enter through stomata of the grass blade (7). Both *R. zeae* (teleomorph: *Waitea circinata* Warcup and Talbot) and *R. oryzae* (teleomorph: *W. circinata*) are responsible for leaf and sheath spot of turfgrasses. Though this is less abundant than brown patch, it has a more or less similar geographical distribution. The *Rhizoctonia* incited yellow patch caused by *R. cerealis* (teleomorph: *Ceratobasidium cereale* Murray and Burpee) is thought to be dominant on annual bluegrass (*Poa annua* L.) in the western United Sates (15). The teleomorph *Waitea circinata* var. *circinata* causing brown ring patch is the latest addition to *Rhizoctonia* diseases on turfgrasses. This disease was initially reported on creeping bentgrass in Japan. In 2003, brown ring patch was first reported in the USA. The new disease appeared in annual bluegrass in eastern Washington State and showed similarity to yellow patch. Unlike yellow patch which is favored by cool weather temperatures ranging from 10-18°C, the brown ring patch symptoms were observed in a wide temperature range of 15-35°C (15). On PDA, *W. circinata* var. *circinata* forms orange to dark brown irregular-shape sclerotia both on the surface and embedded in the

agar plate. Over time, the color of these sclerotia turns dark brown to black. With the emergence of *W. circinata* var. *circinata*, the common teleomorph of *R. zeae* and *R. oryzae* viz. *W. circinata* was also reclassified into two varieties. They are *W. circinata* var. *oryzae* (teleomorph of *R. oryzae*) and *W. circinata* var. *zeae* (teleomorph of *R. zeae*) (46). The leaf and sheath spot disease caused by these two pathogens occurs most frequently during warm and humid environments having a temperature range of 28- 35°C (46). Some of the key characteristics of different *Rhizoctonia* species are summarized in Table 1.1.

Field symptoms of *Rhizoctonia* diseases

Brown patch

Brown patch symptoms can vary with grass type, height of cut and prevailing weather conditions. Mainly cool season turfgrasses are susceptible to this disease. Under close mowing, brown patch produces light brown circular patches of dying and withering leaves from several centimeters to a few meters in diameter. These patches may show a dark purplish or grayish brown border called a smoke ring at the outer perimeter if warm, humid conditions prevail (42). Smoke rings are a result of the pathogen actively growing and infecting grass leaves. Usually smoke rings are observed early in the morning before the dew is dried off the leaves. However, it is not a characteristic feature of this disease and a smoke ring might not occur. There may be a musky odor perceptible 12 to 24 hours before the disease appears (42; 48). Eventually, the individual patches may coalesce to form irregularly shaped areas of blighted grass. Disease severity is high in closely clipped turf such as golf course putting greens. In lawns and athletic fields where cool season grasses are maintained at a height above 6 cm (ex. Tall fescue, Kentucky bluegrass, perennial ryegrass), the primary symptom of brown patch is the light brown circular patches. These patches are typically 15-30 cm in diameter. Sometimes the center grasses of these patches may recover, which eventually lead to a frog eye appearance. Individual diseased leaves have irregular necrotic lesions with a dark brown band of leaf tissue surrounding the lesion (42).

Large patch

Large patch is a destructive disease of warm season grasses especially during spring and autumn. As the name indicates, the patches created by large patch may cover areas ranging from

1-6 m or more in diameter. These patches do not have a smoke ring, but shoots at the margin of the patches may look off-colored during active infection (42). Initially, infected areas may be viewed as light green patches from 5 cm to 0.6 m. When the environment is favorable (i.e. during cool wet and humid weather), these patches rapidly change to bright yellow to orange and then to a brown color (13). Unlike brown patch, leaf lesions are hard to find. Large patch infects the roots and sheath of the grass, causing a sudden death of the entire plant. Infected shoots can be easily detached from the stem. Therefore, therapeutic fungicide application after observation of the symptoms may have little effect on large patch (42).

Yellow patch

Yellow patch is a cool weather disease that thrives at an atmospheric temperature of 10-18°C. This disease mostly affects cool season grasses (ex. annual bluegrass, creeping bentgrass) grown on golf courses and symptoms depend on grass species and climatic conditions (42). Annual bluegrass in golf greens develops distinct tan/straw patches or circles 30 cm to 1 m in diameter. Grasses in the center of large patches often recover leading to a frog-eye pattern. Most of the cool season grasses used for golf fairways, landscapes or sports turf show the same initial symptoms. Symptoms rapidly disappear when weather is dry and temperature increases above 25°C (5). On warm season bermudagrass and zoysiagrass, the disease is limited to leaf yellowing, and leaf necrosis is hardly observed (13).

Leaf and sheath spot

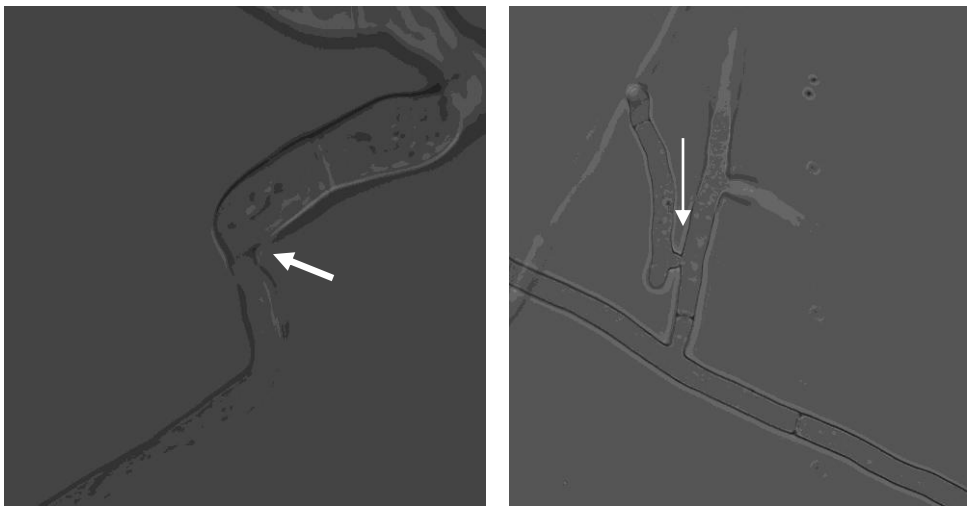
Both *R. zeae* and *R. oryzae* are responsible for this disease. Symptoms are generally very similar to brown patch but can vary depending on the grass species, pathogen, soil conditions and environment. Without *in vitro* culture, it is difficult to identify the causal agent of the disease. In bentgrass *R. zeae*, has been associated with rings or patches colored yellow to orange to brown to gray. In centipedegrass and St. Augustinegrass, *R. zeae* and *R. oryzae* both cause sheath lesions, similar to *R. solani* (42).

Brown ring patch

This disease was first reported in Japan on creeping bentgrass putting greens(15). Symptoms on bentgrass include yellow to brown, circular or irregular patches having a diameter of 10-50 cm. Over time margins of the diseased patches develop a brown ring hence the name

"brown ring patch" has been attributed to this disease. Only turf tissues above the soil surface are reported infected by this pathogen. Most of the time the turf in the center of the patches recovers to produce a "frog eye" (46). In the United States this disease has been identified from annual bluegrass and rough bluegrass. At present, the disease has been reported from western, Midwestern and eastern USA. Symptoms on annual bluegrass are very similar to yellow patch (15). Typically, disease initially appears as thin yellow rings having a diameter of several centimeters to 1 m. Rings are either circular or irregular in shape. These rings start out yellow and turn brown and necrotic over time. Though yellow patch is limited to cool temperatures, brown ring patch generally becomes worse with increasing temperature (>28° C).

Figure 1.4 Perfect anastomosis reaction of *R. solani* hyphae. The arrows indicate hyphal fusion points.



Traditional method of grouping *Rhizoctonia* species

Rhizoctonia species in general are not well understood due to morphological similarities and lack of information about mating and mating relationships (29). The most important *Rhizoctonia* species, *R. solani,* is a species complex, consisting of divergent populations. The genetics of *R. solani* is currently not well defined. There are subgroups within this complex and traditionally focus has been on the anastomosis reaction of the isolates. Anastomosis is the hyphal fusion reactions of vegetatively compatible isolates. Anastomosis groups (AGs) also

represent genetic isolation and therefore isolates belonging to the same AG are genetically closer than isolates of different AGs. The hyphal fusion reaction has different levels or categories. Typically, hyphae of genetically similar isolates fuse perfectly. A perfect fusion usually results in exchanging cell contents. Carling et al. categorized hyphal fusion reaction into four groups (10). If there is no hyphal reaction between two strains, it is designated as C0 and the two isolates belong to different AGs. C1 represents hyphae of two isolates growing towards each other and contact of cell walls. However, cell walls of these contact points do not fuse. The isolates of this type of reaction may have a more distant relationship within an AG and may be members of highly heterogeneous AGs such as 2, 8 or BI. A C2 type reaction represents related isolates that are in the same AG but in different vegetatively compatible sub-populations. In a C2 reaction the hyphal cell walls not only contact but also fuse. However, no cytoplasmic fusion is observed because cell wall fusion is followed by cell death. The fourth and final reaction type C3 represents perfect hyphal fusion (Figure 1.4) where both cell wall and cell membrane of the hyphae of opposing isolates fuse. This is considered true anastomosis with the possibility of exchanging cell contents. By definition, observation of five or more anastomosis points in three replicates in the C1, C2 or C3 category permits the allocation of tested pair to that category. Currently, based on hyphal anastomosis reactions, *R. solani* strains have been divided into 14 AGs (Table 1.2) (29). Seven of the 14 AGs have been divided into subgroups to reflect differences observed in the culture appearance, morphology, host range, pathogenicity, thiamine requirements and hyphal fusion frequency. Some of the subgroups are further divided into groups or types (7).

Rhizoctonia solani isolated from turfgrasses thus far have been assigned to six anastomosis groups: AG 1, AG 2-2, AG 3, AG 4, AG 5 and AG 6 (54). The isolates that fall into different AGs may represent specific turfgrass species, a plant part or probably a geographic region. For example Martin and Lucas reported most of the isolates collected from foliage of cool season grasses (mainly tall fescue) in Raleigh, North Carolina belonged to AG 1 (34). Isolates of diseased leaf sheaths from the warm season St. Augustinegrass in Texas and South Carolina have been identified as *R. solani* AG 2-2 (7). Though isolates of *R. zeae* from rice, millet, pine and soil, and *R. oryzae* from rice and soil have been assigned to *Waitea* anastomosis groups WAG-Z and WAG-O respectively, turfgrass isolates of *R. zeae and R. oryzae* have not shown any affiliation to these WAG groups (7). The isolates of binucleate *Rhizoctonia* species

(ex. *R. cerealis*) with *Ceratobasidium* teleomorphs consist of at least 17 AGs spanning from AG-A to AG-Q (Japanese groups) (7). These Japanese AG groups correspond to *Ceratobasidium*

Table 1.1 Summary of key features of *Rhizoctonia* species of turfgrasses (7; 42; 46)

Anamorph	Teleomorph	Temperature range (C°)	Colony color	Sclerotium color	Anastomosis group/s
R. solani	*Thanatephorus cucumeris*	21-32	buff to brown	dark brown	AG 1-1A, -IB AG 2-2IIIB,-2 LP AG 3, AG 4, AG 5, AG 6
R. cerealis	*Ceratobasidium cereale*	10-18	white to buff	light to dark brown	AG-D (CAG-1)
R. zeae	*W. circinata* var. *zeae*	28-35	white to buff to salmon	orange	WAG-Z
R. oryzae	*W. circinata* var. *oryzae*	28-35	white to buff to salmon	salmon	WAG-O
None	*W. circinata* var. *circinata*	15-35	white to orange to dark brown	orange to dark brown	none

anastomosis groups (CAG 1 to CAG 7) established by Burpee in North America (7). The CAG groups were subsequently incorporated into the AG system developed in Japan. However, CAG 5 and 7 were designated as new AG-R and AG-S respectively (41). *Rhizoctonia cerealis* isolated from turfgrass causing yellow patch symptoms belongs to a single group of AG-D or CAG-1. However, isolates causing yellow patch symptoms in Japan have been assigned to AG-Q (7). There are numerous reports of binucleate *Rhizoctonia*-like fungi (BNR) being isolated from turfgrass swards or soils. These differ from *R. cerealis* in morphological or physiological features and anastomosis reactions. Pathogenicity tests conducted on a few occasions have revealed BNR to be non pathogenic or weakly virulent on turfgrasses (6; 7; 20). In comparison to isolates of *R. solani*, hyphal anastomosis reactions of BNR from turf have not been characterized well (7). Although isolates in the same AGs are thought to be more genetically homogeneous

than isolates in different AGs, sometimes they fail to anastomose. The reason behind this may be genetic instability, environmental, or nutrient conditions (29). Furthermore, the fusion reaction

Table 1.2 List of *Rhizoctonia* anastomosis groups (AGs) and AG subgroups[a] and main hosts

AG	Subgroup	Hosts
AG 1	AG 1-1A	Rice, corn, sorghum, bean, soybean, crimson clover, camphor seedlings, turfgrasses
	AG 1-IB	Sugar beet, rice bean, soybean, snap bean, corn, cabbage, lettuce, turfgrasses
	AG 1-1C	Sugar beet, buckwheat, carrot, flax, soybean, pine
	AG 1-1D	Coffee
AG 2	AG 2-1	Pea, crucifers (cabbage, radish, broccoli), strawberry, tulip, subterranean clover, lettuce
	AG 2-2IIIB	Rush, rice, ginger, gladiolas, edible burdock, corn, sugar beet, chrysanthemum, Chinese yam, turfgrasses
	AG 2-2IV	Sugar beat
	AG 2-2LP	Warm season turfgrasses (bermudagrass, Zoysiagrass)
	AG 2-3	Soy bean, bean
	AG 2-4	Carrot, corn, lettuce, radish, sugar beet, cauliflower
AG 3	AG 3 (PT, TB)	Potato, tomato, tobacco, egg plant, sugar beet seedlings
AG 4	AG 4 HG (I, II, III)	Peanut, sugar beet, pea, spinach, potato, onion, snap bean, tomato, melon, turfgrasses
AG 5		Soybean, potato, bean, turfgrasses
AG 6	AG (6-I, 6-V)	Turfgrasses (weak pathogen)
AG 7		Soil (may be non pathogenic)
AG 8		Winter and spring wheat, spring barley, yellow mustard, and safflower.
AG 9	AG 9 (TP, TX)	Crucifers and potato, carrot, lettuce (mildly parasitic)
AG 10		Barley, basal rot of lettuce, tomato (weak pathogen)
AG 11		Rice
AG 12		Many of its isolates are mycorrhizal with the Australian orchid *Pterostylis acuminata*
AG 13		Cotton, radish, cauliflower (weak pathogen)
AG BI		Soil (non pathogenic)

[a]Extracted from refs. (28; 29; 43)

may be difficult to interpret. The fungus occasionally can exhibit different types of anastomosis reactions for isolates of the same AG. The existence of bridging isolates sometimes makes placement of an isolate into a relevant AG a difficult task. Bridging isolates are isolates that

apparently fuse with isolates of another AG (8). For example, AG 8 and AG BI have shown bridging reactions with some isolates of AGs belonging to 2, 3, 6 and 11 (8). This hyphal fusion is imperfect where only cell walls of hyphae connect with no membrane contact (8). Under these circumstances AGs themselves may not necessarily give information on the genetic variation and taxonomic relationships between AGs (8; 29). This has led to the necessity of developing additional identification methods which could supplement anastomosis reactions. It is also important to note that isolates of different subgroups within an AG are not distinguishable on the basis of anastomosis reactions. The only exception may be isolates of AG 2-1 and AG 2-2 because of their high genetic diversity (8).

Molecular methods used for characterization of *Rhizoctonia* species

DNA-DNA hybridization

One of the earliest molecular methods used to identify *Rhizoctonia* species is DNA comparisons and DNA-DNA hybridization experiments. Kuninaga and Yokosawa (25; 26), and Vilgalys (49) carried out experiments to resolve genetic relatedness among and within AGs of *Rhizoctonia solani* isolates through DNA comparisons. Kuninaga and Yokosawa found the GC content of the isolates within each AG to be very similar. However, the *R. solani* complex was found to have a wide variation in GC content which range from 43.2 to 49.5 % (24). A study by Kurtzman in 1987 on fungal strains had concluded that different isolates differing by GC contents in excess of 2.0-2.5 % likely belonged to different species (24). Though a wide range of GC content values found in *R. solani* indicates a possibility of AGs representing different species, genomic DNA-DNA hybridization technique based separations provide more conclusive evidence to support this concept. The degree of reassociation (hybridization) between DNA molecules of different microbial isolates provides a measure of genetic relatedness (29). DNA reassociation could be carried out either in a free solution or when one of the DNA species is immobilized on a filter. The DNA reassociation is monitored spectrophotometrically by measuring the kinetics of duplex formation, or by tagging the molecules with radioactivity (29).

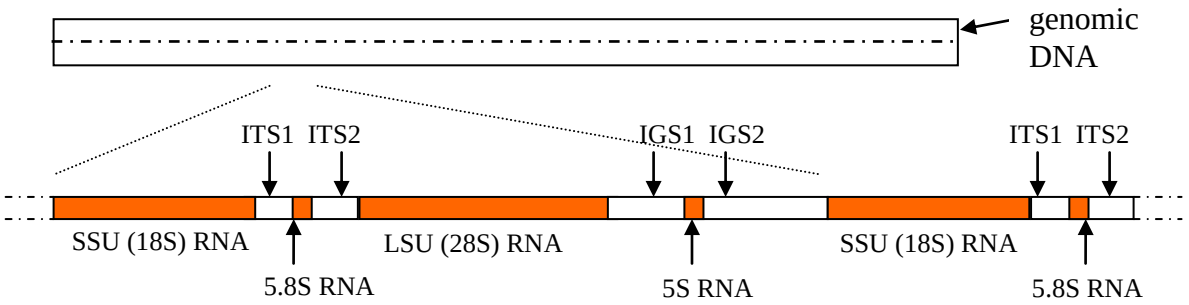
DNA-DNA hybridization values obtained for isolates within most of the AGs were very high (≥ 90%) whereas isolates corresponding to different AGs showed low association and therefore low DNA relatedness (29). In AGs 2-1, 3, 5, 7, 8 and BI, the members of the same AG hybridized with each other at a rate of ≥ 91%. The hybridization studies also showed

considerable genetic differences among subgroups of the certain AGs, viz. 1, 2, 4, 6 and 9 (29). This low relatedness of AG subgroups was compatible with previously identified subgroups based on cultural morphology, pathogenicity and vitamin requirement (24). Between different AGs, the hybridization value is normally less than 15% (29). The hybridization value of subgroups within an AG is mostly less than 60%. The genomic DNA-DNA hybridization experiments not only ascertain the genetic relatedness but also may reveal the previously unknown heterogeneity. New subgroups of AG 4 and AG 6 were observed by this method. The hybridization values between members of AG 4-HGI and HGII were 30 to 47% while the hybridization values between isolates of AG 6 subgroups HGI and GV were 47%-62% which confirm their affiliation to separate subgroups (24). The DNA relatedness in AGs of the *R. solani* complex representing distinct evolutionary units corresponding to separate species was termed as "genomic species" by Kuninaga (24). Time consuming pair wise comparison is a main disadvantage of DNA-DNA hybridization technique. Additionally it requires the entire genome of the species to conduct the experiment. At present the DNA hybridization technique has been replaced by newer, easier methods such as polymerase chain reaction (PCR) based fingerprinting techniques (29).

Analysis of ribosomal RNA genes

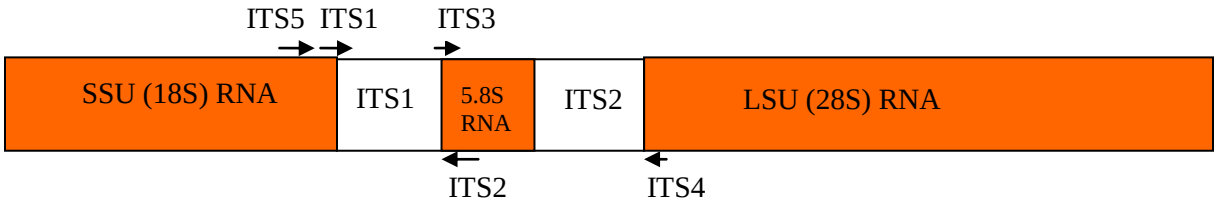
The DNA sequences that encode ribosomal RNAs have been extensively used in comparative analysis for taxonomic and phylogenetic studies in fungi (14). The ribosomal genes (rDNA) of fungi are located in either mitochondria or nuclei. There are regions with highly conserved and highly variable sequences within the ribosomal DNA. These genes of fungi are arranged as a multiple copy series in tandem units that contain DNA sequences for 18S (small), 5.8S and 28S (large) rRNA subunits (Figure 1.5). The 5.8S subunit is flanked by two internal transcribed spacer regions known as ITS1 and ITS2. RNA subunits 18S and 28S are located outside of ITS1 and ITS2 respectively. The repeats of 18S-5.8S-28S rDNA regions are separated by intergenic spacer (IGS) regions and these normally contain the gene for 5S rRNA subunit (14). An experiment carried out by Vilgalys and Gonzales found *Thanatephorus praticola* (anamorph: *R. solani* AG 4) rDNA repeats to have a length of 8.8 kb and the estimated number of rDNA copies to be 59 per haploid genome (50).

Figure 1.5 The structure of fungal nuclear ribosomal DNA repeat unit. The internal transcribed spacer (ITS) region is flanked by 18S (SSU) and 28S (LSU) rRNA subunits. The ITS region consists of ITS1, 5.8S RNA and ITS2 sub regions. 18S and 28S rRNA subunits are separated by intergenic spacer region (IGS).



Polymerase chain reaction generated rDNA-RFLP (Restriction fragment length polymorphism) has been used to analyze the rDNA gene of *Rhizoctonia* isolates since the early 1990s (40). In this method, initially genomic DNA is amplified with specific primers to obtain multiple copies of the rDNA region. Then the amplified rDNA products are digested with more than one restriction enzyme and resulting DNA fragments are separated based on their size by gel electrophoresis. Comparison of these restriction patterns can demonstrate genetic differences of unknown isolates. The polymorphism variations of rDNA-RFLP enabled the establishments of new subgroups within the existing *R. solani* AGs such as in AG 2-3 (bean foliar blight pathogen), and AG 1-ID (coffee necrotic leaf spot pathogen) (40). Another study conducted in India by Taheri et al. demonstrated rDNA-ITS-RFLP can be used to differentiate isolates of *R. solani* of rice to their anastomosis subgroup level (45). They used *Mun*I and *Mse*I endonucleases for restriction digestion of PCR products to generate unique banding patterns for AG subgroups. However, rDNA-RFLP can give erroneous results due to mutations such as insertions and deletions (indels). The presence of such "indels" to the length of even a single base may result in changes in more than one fragment. As the number of fragment differences is used to estimate the degree of nucleotide divergence, the accuracy of the conclusions may be affected (40).

Figure 1.6 Location of several nuclear ribosomal DNA internal transcribed spacer (rDNA-ITS) primers.



Since the mid 1990's sequencing of the rDNA- ITS region has become extremely popular for phylogenetic analyses and population studies of isolates of *Rhizoctonia* (40). This method can overcome most of the weaknesses of the rDNA-RFLP technique mentioned above. According to Lübeck (29), the majority of *R. solani* sequences present in publicly available databases consists of rDNA sequences. This is an indication of reliance on rDNA sequence analyses for phylogenetic studies of *R. solani*. Most of the available rDNA sequences are comprised of sequences of the ITS region (ITS1-5.8S-ITS2). Furthermore, sequences of 18S and 28S ribosomal RNA subunit regions are also available for this pathogen in publicly available databases. Availability of such sequences not only opens up more avenues to investigate phylogenetic relationships but also for identification purposes of *R. solani* isolates derived from different host species. Another advantage of ITS sequencing is the ready availability of well tested primer pairs. Figure 1.6 shows the locations of several fungal ITS primers designed by White et al. (52) and Table 1.3 shows the nucleotide composition of these primers. Sharon et al. in their review paper on *Rhizoctonia* stated that at present, analysis of rDNA sequence data seems to offer the most accurate way of deriving phylogenetic and taxonomic relationships for these species (40).

Table 1.3 Primers for amplification of nuclear internal transcribed spacer (ITS) region (adapted from White et al. (52))

Primer name	Primer sequence (5´-3´)	T_m(°C)
ITS1	TCCGTAGGTGAACCTGCGC	65
ITS5	GGAAGTAAAAGTCGTAACAAGG	63
ITS2	GCTGCGTTCTTCATCGATGC	62
ITS3	GCATCGATGAAGAACGCAGC	62
ITS4	TCCTCCGCTTATTGATATGC	58

T_m: melting temperature

According to previous studies, the 5.8S region of *R. solani* rDNA is highly conserved. However, ITS regions flanking the 5.8S region show high variability among genetically different isolates since these sequences are not coding and therefore are subjected to less evolutionary pressure. This sequence variability can be used as a valuable tool for identifying AGs and AG subgroups of biological significance (10; 18; 27). Kuninaga et al. examined ITS regions of rDNA in 45 isolates (from different host species) representing 11 AGs and 11 AG subgroups of *R. solani* (27). They found the sequence homology of ITS regions for isolates of the same subgroups was above 96%. Isolates of different subgroups within an AG had sequence similarity of 66-100%. Sequence similarity of isolates of different AGs varied from 55-96%. Among the isolates analyzed, the sequence of ITS1 was more variable than ITS2 region.

The rDNA sequence analysis carried out so far has confirmed the genetic differences of *Rhizoctonia* isolates of different AGs and AG subgroups. Therefore, studies on rRNA genes support the traditional anastomosis grouping. Similar results were obtained by DNA-DNA hybridization, rDNA-RFLP analysis and other molecular methods (29). Although AGs and subgroups within AGs show genetic variability, there have been reports that express uncertainty about taxonomic placement of certain AGs of *Ceratobasidium* and *Thanatephorus* genera. This is due to the observation of hyphal fusion among isolates of AG 6 (*Thanatephorus*) with AG-F (*Ceratobasidium*) (29). An analysis done by Gonzalez et al. revealed certain AGs are not monophyletic and that there is a greater taxonomic support for AG subgroups than for AGs (18). According to them the most fundamental evolutionary unit within *Thanatephorus* is not AGs but AG subgroups.

PCR fingerprinting techniques

Prior to the 1990s, DNA characterization employed the Southern blot technique or selective DNA amplification. Prior knowledge of DNA sequences, characterized probes or clones was a prerequisite for these methods (47). The polymerase chain reaction (PCR) based techniques developed afterwards require relatively short (5-20 nucleotides), arbitrary oligonucleotide primers to obtain DNA polymerase mediated amplification of discrete but anonymous DNA segments. Random amplified polymorphic DNA (RAPD) analysis and DNA amplification fingerprinting (DAF) are two common techniques among many which employ the principle of PCR. The main difference of conventional PCR from PCR fingerprinting techniques

such as RAPD and DAF is that conventional PCR uses two primers targeting known target sites and amplify one fragment of known or predicted size (31). Conversely, the PCR fingerprinting techniques such as RAPD and DAF utilize a single primer to obtain multiple copies of DNA fragments of variable sizes. Size variability of these fragments depends on the primer used and characteristics of the genome under investigation. Initially, arbitrary oligonucleotide primers used in fingerprinting methods bind to many sites of the genomic DNA template and synthesize complementary nucleotide fragments of various sizes. In successive PCR cycles, previously synthesized strands serve as template DNA during the annealing step. However, amplification takes place selectively only in genomic regions bordered by primer annealing sites occurring in opposite strands and separated by a few thousand bases. The main difference between DAF and RAPD is the primer length. DAF has primers of 5-8 nucleotides whereas RAPD primers consist of 10 nucleotides. Normally, RAPD results in fewer bands compared to DAF. Separation by agarose or polyacrylamide gel electrophoresis and staining with ethidium bromide, silver, SYBR gold or detection using radionucleotide or biotinylated DNA are a few examples (31). The DNA fragments specific to a strain can be considered as genomic "fingerprints" and the polymorphism of fragments among different strains can be detected in the DNA banding profiles (29). Recently PCR fingerprinting techniques have been used to assess genetic differences of AG subgroups of *R. solani* (11; 38; 44).

Universally primed PCR (UP-PCR) is another DNA fingerprinting method very similar to RAPD method. The main difference between UP-PCR and RAPD is the length of the primers used. These primers are longer (15-21 nt) than conventional RAPD primers (typically 10 nt) and designed to anneal under more stringent conditions (52– 60°C) ensuring reproducibility of UP-PCR banding profiles (31; 47). In contrast RAPD uses lower annealing temperatures (35-40°C) where the banding patterns have been shown to be less consistent even when the experiment is repeated in the same laboratory. The universal primers (UP) were originally designed to target and multiply intergenic, phylogenetically less conserved regions of genomes (31). The UP-PCR generated fragments resulted by amplification of intergenic regions are species specific and some of these bands have the potential to act as probes or markers for the fungi tested. Also depending on the fungus, some highly polymorphic amplified fragments may enable detection of infra-specific variation (3). This ability to distinguish among closely related strains is due to the reliable generation of a higher number of bands (60-100) including weak bands which in total

represent approximately 10^4 nucleotide base pairs randomly covering the genome. In contrast, the complexity of DNA band profile generated by RAPD protocols is smaller.

Though PCR fingerprinting methods give an indication of genetic relatedness by generating similar banding profiles for genetically similar strains, these methods cannot be used to derive phylogenetic relationships among different clades of a tree (31). Similar banding profiles or fragments can also be produced by genetically unrelated strains or species. Due to this difficulty of determining common descent from co-migrating fragments, bands generated from PCR fingerprinting techniques are considered as phenetic or observed characters rather than phylogenetic (11; 31). Advancement of technology has helped the researchers to analyze PCR bands digitally with the help of a genetic analyzer. The primers for this purpose need to be tagged with a fluorescent dye such as 6-FAM or HEX. The fluorescence-tagged DNA fragments are sent through a capillary filled with a special polymer which acts as a separation matrix. These separated fragments are digitally recorded as peaks. These analyzers can detect more bands with less sample volume in comparison to the traditional gel electrophoresis. Powerful computer software programs have been developed to convert peaks into a binary table of zeros and ones which can be used for constructing dendrograms and cladistic analyses.

Various PCR fingerprinting techniques use the same principle of DNA polymerase mediated amplification of DNA fragments to generate multiple copies of target genome sites. The differences of these techniques lie primarily in the design or choice of primers and level of stringency. However, the more recent technique, amplified fragment length polymorphism (AFLP) (51) differs from the above methods. In AFLP, prior to amplification, the genomic DNA is digested with two restriction endonucleases followed by attaching double stranded oligonucleotide adapters to the fragments to generate template DNA for amplification (29; 31; 51). Two primers recognizing the adapter sequences and adjacent restriction site are used for the amplification process based on PCR. The amplification products are separated by running through a denaturing polyacrylamide gel and visualized by ultraviolet light after staining with ethidium bromide (29; 51).

In AFLP, restriction fragment amplification is done in two steps: a pre-amplification step and a selective amplification step. The primers of pre-amplification contain the sequence of the adapters and may also include an arbitrary nucleotide to limit the number of fragments amplified. The selective amplification step utilizes primers with 2-3 arbitrary nucleotides in addition to the

adapter sequence. Selective nucleotides are included at the 3' ends of the PCR primers. Therefore, only restriction fragments in which the nucleotides flanking the restriction site match the selective nucleotides will be amplified (51). The restriction enzymes used in AFLP commonly employ a rare cutter and a frequent cutter such as six base *Eco*RI and four base *Mse*I, respectively. The frequent cutter generates small DNA fragments, which will amplify well and are in the optimal size range for separation on denaturing gels while the rare cutter reduces the number of fragments to be amplified. Additionally, the number of bands can also be controlled by selective bases in the primers. Selective extensions with rare trinucleotides can limit the number of fragments significantly. According to Vos et al. a complex genome digested with a single enzyme combination (a combination of a specific six-base and four-base restriction enzyme) may permit the amplification of 100,000s of unique AFLP fragments, of which generally 50-100 are selected for each AFLP reaction (51).

Majer et al., who tested AFLP to detect inter- and intraspecific genetic variation of two fungal pathogens *Cladosporium fulvum* and *Pyrenopeziza brassicae*, found that the technique was very efficient at identifying polymorphisms even in species where very little variation could previously be found by RFLP analysis (33). The number of bands generated from AFLP was in the range of 50-70. The advantages of this method are higher reproducibility and higher proportion of the genome being analyzed per reaction.

UP-PCR product cross-hybridization

Cross-hybridization of UP-PCR products is another dimension of the UP-PCR method. Cross-hybridization of UP-PCR products derived from a single UP primer can be used to determine the sequence similarity (homology) of unknown *Rhizoctonia* strains. This facilitates the identification of UP-PCR hybridization groups (31). The UP-PCR products from different strains are first blotted on to a membrane and labeled UP-PCR products of a reference strain are used as a hybridization probe in each blot. One advantage of this method is the ability to investigate sequence homology of many strains simultaneously. Probe DNA can be labeled by radioactive phosphorus or a non-radioactive molecule such as digoxigenin (DIG). The strength of the hybridization signal is used to determine the relatedness of the strain to the unknown one. If radioactive probes are used, strong signals may be visible on an autoradiograph after one hour. These signals indicate the hybridized strains belong to same UP-PCR hybridization group. Weak

signals visible after a few hours or more demonstrate strains that are related to a lesser degree. No signal after overnight exposure reveals the absence of any relationship of the strains tested. Non-radioactive detection methods are based on antigen- antibody reactions. Once the probe is attached to immobilized unknown UP-PCR products, excessive probe is washed off and anti-digoxigenin solution is introduced to the membrane. Often one end of these DIG antibodies are attached with alkaline phosphatase (AP) enzyme which can breakdown certain substrates such as either BCIP/NBT (5-Bromo-4-chloro-3-indolyl phosphate/ nitro blue tetrazolium chloride) to produce a color. Anti-DIG-AP conjugate can also breakdown chemiluminescence substrates such as dioxetane to emit a light. In other words these non-radioactive detection methods are based on either colorimetric or chemiluminescent reactions. In particular chemiluminescence detection methods can be very sensitive and may need only few minutes of exposure to an autoradiography film to visualize the signal of homologous UP-PCR products. Universally primed PCR products represent only a partial image of the genome in contrast to DNA-DNA hybridization, in which the whole genome is screened. As mentioned earlier, UP-PCR fragments like any set of fragments generated by PCR fingerprinting techniques represent phenotypes. However, UP-PCR product cross-hybridization allows deriving sequence homology and therefore has a phylogenetic dimension.

Lübeck and Poulsen used a UP-PCR cross-hybridization assay for rapid identification of *Rhizoctonia* isolates into their AGs (30). Twenty one tester isolates belonging to 11 AGs of *R. solani* were exposed to UP-PCR with a single UP primer and the amplified products were spotted on to a filter, immobilized and used for cross-hybridization against amplification products from different isolates. They found isolates within AG subgroups cross-hybridize strongly whereas different AG subgroups and AGs showed little or no cross-hybridization (30). Furthermore, sixteen *Rhizoctonia* isolates from diseased sugar beets and potatoes were identified using the assay. The visual comparison of UP-PCR banding profiles of tester isolates with unknown *R. solani* isolates obtained from diseased potato and sugar beets in many occasions revealed their identification. This differentiation into subgroups was possible because there were many bands shared among isolates within an AG subgroup. Lübeck and Poulsen found that UP-PCR hybridization data were compatible with total DNA-DNA hybridization data (30). A strong UP-PCR cross-hybridization value (signal within one hour with radioactive labeling) seems to indicate a DNA hybridization value of more than 75%. A significant signal (signal within 12

hours) may represent a DNA hybridization value of approximately 60-75%, and a weak signal (signal after 2-3 days) indicates a DNA hybridization value of approximately 40-60%. No UP-PCR cross blot hybridization value was obtained with DNA hybridization values of less than 40% (30).

UP-PCR derived SCAR markers

The PCR fingerprinting techniques require pure cultures of the strains under investigation to produce meaningful results. The primers used in these methods are capable of amplifying DNA fragments from any organism and therefore cannot be used to detect a specific species or a strain from soil or infected plant parts (31). However, by identifying unique molecular markers present in the target organisms, the fingerprinting methods including UP-PCR can be used for rapid identification of organisms of interest. The essential feature of the marker is to detect all members of the target group and discriminate against all others. When such markers are sequenced they are called sequence-characterized amplified regions (SCARs) (31). The sequence information of the SCAR markers can be used to synthesize primers that selectively amplify the marker in identifying the target organism/s in diagnostic assays. *Rhizoctonia* blight is caused by different *Rhizoctonia* species and AGs having differential sensitivity to fungicides. Optimum climatic conditions for disease development also differ for different species and AGs. Therefore rapid identification of the causal agents of the *Rhizoctonia* blight is important for disease management. SCAR markers need to be identified at AG subgroup level in order to use this technique in a meaningful way. However, UP-PCR derived markers for strains of a subgroup may not be available.

As an example, UP-PCR method has been used to selectively recognize a single antagonistic strain of *Clonostachys rosea* (syn. *Gliocladium roseum*) which is a well known mycoparasite of many soil borne pathogens (4). The antagonists were screened by UP-PCR and subsequently, a strain specific marker was identified for the strain GR5. The marker was converted into a SCAR and a specific primer pair was designed for selective amplification of GR5. Eighty two strains and DNA from 31 soil samples, mostly of Danish origin, were tested for specificity of SCAR markers. Out of 82 strains two responded to SCAR primers which were similar to GR5 but not identical. The total DNA extracted from soil samples infested with and

without GR5 demonstrated the SCAR primers could detect GR5 in a pool of mixed DNA and that this particular marker was not present in other microorganisms.

Differential resistance to fungicides by *Rhizoctonia* species and isolates

Many turfgrasses are susceptible to all the above mentioned species of *Rhizoctonia* and are often infected by several species simultaneously. Within *R. solani*, six genetically distinct anastomosis groups have been reported to cause brown patch in turfgrass (54). In case of multiple infections, successful control of *Rhizoctonia* blight requires fungicides with activity against all species and genetically isolated populations. For golf greens and fairways an application of fungicides is a common and routine practice to control *Rhizoctonia* diseases. Athletic fields and home lawns also employ fungicides, but to a lesser extent in managing brown patch diseases. *In vitro* studies have indicated differential responses by isolates and species to most of the recommended fungicides (7; 21; 22). Since it is unlikely to find a single broad spectrum fungicide to control all *Rhizoctonia* species and isolates, adequate control of this disease complex requires not only accurate identification of the pathogen but also information on the effectiveness of recommended fungicides.

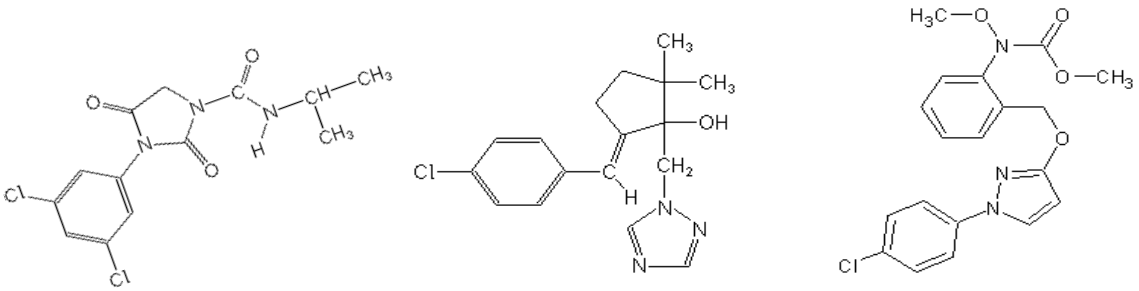
This study included *in vitro* evaluation of three commonly used fungicides against *Rhizoctonia* diseases. They are iprodione, triticonazole and pyraclostrobin. These fungicides belong to chemical classes with different modes of action. The chemical structures of these fungicides are given in Figure 1.4.

Iprodione belongs to the dicarboximide group and was introduced to the market three decades ago to control a wide variety of fungal diseases including brown patch of turfgrass. It is also used to control different diseases in root crops, ornamentals, vegetables, and cotton (39). Despite years of study, the precise mode of action of dicarboximide fungicides is still unclear. However, the latest studies have revealed these fungicides to show their antifungal properties by over-activating osmotic signal transduction of MAPK (mitogen-activated protein kinase) downstream of OS-1 (osmotic stress sensitive) histidine kinase (16; 37). Sensitive fungal cells produce osmotic stress responses leading to swelling and bursting of the cells. Iprodione has only a translaminar movement and not efficiently transported through vascular tissues. Previous studies show that *R. solani* and binucleate *Rhizoctonia* are more sensitive to iprodione than are *R. zeae* and *R oryzae* (9; 22; 35; 36).

The second tested fungicide, triticonazole, is a systemic fungicide with a triazole moiety having acropetal movement. Triticonazole is a DMI (de-methylation inhibitor) fungicide that disrupts sterol biosynthesis in fungal cell membranes, and leads to alterations of the structure and disturbances in the division and development of cells. Triticonazole was introduced by Rhone-Poulenc Ag company in the mid 1990s (2). Studies on other DMI fungicides such as propiconazole, fenarimol, and triadimefon have shown variable efficacy on different species and AGs of *Rhizoctonia* and therefore the success of their use in managing *Rhizoctonia* diseases in turf has been variable (21; 48).

Pyraclostrobin is a relatively new fungicide for turfgrass disease control. It was first marketed in 2002 by BASF chemical company (1). This fungicide is highly recommended for control of brown patch, large patch and leaf and sheath spot diseases of turfgrasses. Pyraclostrobin belongs to the fungicide group of quinone outside inhibitors (QoI) or strobilurins. It inhibits mitochondrial respiration within fungal cells. Since the electron transport at the cytochrome bc-1 complex is blocked, fungal cells that are exposed to these molecules are starved of energy (ATP) required for disease development, and ultimately growth is restricted (1; 17). Pyraclostrobin has a translaminar movement in plants. No records of resistance have been reported in *R. solani* or *Waitea* species to this fungicide. However, pyraclostrobin is a single-site fungicide since it blocks only one metabolic pathway of the pathogen. Therefore, only a single mutation in the pathogen is needed for resistance to develop. As development of resistance to this type of fungicides can be rapid compared to fungicides with multisite modes of action, an investigation may be useful. Several QoI fungicides have shown reduced effectiveness for some other fungal pathogens due to site-specific mutations (12; 23; 32).

Figure 1.7 Chemical structures of iprodione, triticonazole and pyraclostrobin (left to right).



Iprodione Triticonazole Pyraclostrobin

This project was undertaken to evaluate the presence and diversity of *Rhizoctonia* pathogens causing disease on turfgrasses. Of the 5 anamorphic species (each with different AGs, some multiple AGs) known to cause disease in turfgrass we expected to find several of these species at each sampling location. Based on preliminary, unpublished data (David McCall, personal comm.) there was also a possibility that the diversity of organisms at each location may be different or specialized. Therefore, we sampled multiple locations in order to address this possibility.

However, it became apparent that AG was not sufficient to identify the various pathogens present at particular locations, and the classical techniques were time consuming, and inefficient. Adequate molecular identification techniques did not exist to identify turfgrass *Rhizoctonia* pathogens to subgroup level, and thus techniques had to be adapted and developed from other *Rhizoctonia* pathosystems. Thus, our primary hypothesis for this study was that *Rhizoctonia* isolates infecting turfgrasses were represented by at least 5 known species, and multiple AGs could potentially occur simultaneously in the same sward/site. This hypothesis was tested using the following specific objectives:

Objectives

1. Group *Rhizoctonia* isolates into species and anastomosis groups using classical methods.

2. Group *Rhizoctonia* isolates cladistically using multiple molecular techniques including; the sequence of the internal transcribed spacer region of ribosomal DNA (rDNA-ITS), universally primed polymerase chain reaction (UP-PCR), and amplified fragment length polymorphism (AFLP) to compare with groups derived using classical techniques.

3. Compare classical and molecular identification methods to determine if molecular methods are able to identify unknown isolates consistent with their classical identification.

Once isolates can be identified using molecular techniques, it is important to be able to efficiently identify unknown isolates to a particular species, AG, or AG subgroup. The ability to rapidly identify isolates is the major impediment to being able to address our initial goal;

determining if there is geographic specialization among various *Rhizoctonia* species, AGs, and AG subgroups. Another hypothesis of this project was that molecular techniques that have been shown to efficiently identify unknown *Rhizoctonia* isolates could also be used to identify unknown *Rhizoctonia* isolates from turfgrass. This hypothesis was tested using the following specific objective:

4. Develop and use two molecular techniques, namely, UP-PCR product cross-hybridization and sequence-characterized amplified region (SCAR) markers to rapidly, and accurately identify unknown *Rhizoctonia* isolates to their appropriate species, AG, or AG subgroup level.

Fungicide activity has also been shown to vary with respect to the various *Rhizoctonia* pathogens that cause disease on turfgrass. However, some of the more recently identified pathogens (i.e. *Waitea circinata* var. *circinata*) have not been screened for variable fungicide control. As a result the final hypothesis of this project was that *in vitro* fungicidal control of various *Rhizoctonia* pathogens would be variable based on the species, AG or AG subgroup tested. This hypothesis was tested using the following specific objective:

5. Screen and calculate the EC_{50} (effective concentration for 50% growth inhibition) of various *Rhizoctonia* isolates to determine the relative efficacy that various fungicide modes of action (e.g. dicarboximide, demethylation inhibitor, and strobilurin) have on different isolates.

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CHAPTER 2

Characterization of *Rhizoctonia* isolates from turfgrasses in Virginia and Maryland using ITS sequencing, UP-PCR, and AFLP fingerprinting

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ABSTRACT

Rhizoctonia blight (sensu lato) is a common and serious disease of many turfgrass species. The most widespread causal agent, *R. solani*, consists of several genetically different subpopulations. Though hyphal anastomosis reactions have been used to group *Rhizoctonia* species, they are time consuming and sometimes difficult to interpret. Isolates of different subgroups within an anastomosis group (AG) are not distinguishable based on anastomosis reactions since they anastomose each other. This study evaluated molecular techniques in comparison with traditional anastomosis grouping to identify and group isolates of *Rhizoctonia*. More than 400 *Rhizoctonia* isolates were collected from diseased turfgrass leaves from eight geographic areas in Virginia and Maryland. A random sample of 86 isolates was selected and initially characterized by colony morphology, nuclei staining and anastomosis grouping. Molecular identification was performed by analysis of rDNA-ITS region and DNA fingerprinting techniques universally primed PCR (UP-PCR) and amplified fragment length polymorphism (AFLP). The cladistic analysis of ITS sequences and UP-PCR fragments supported seven clusters. Isolates of *R. solani* AG 1-IB (n=18), AG 2-2IIIB (n=30) and AG 5 (n=1) clustered separately. *Waitea circinata* var. *zeae* (n=11), and var. *circinata* (n=4) grouped separately. A cluster of six isolates (UWC) did not fall into any known *Waitea* group. Most of the binucleate *Rhizoctonia*-like fungi (BNR) (n=16) grouped separately. AFLP grouping also largely agreed with the above results. However, UWC isolates clustered into two groups. Molecular analyses corresponded well with traditional anastomosis grouping by clustering isolates within an AG or AG subgroup together. Our study indicated that several *Rhizoctonia* species and AGs are responsible for causing patch diseases on cool-season turfgrasses. Results of the molecular analysis also suggest that more than one *Rhizoctonia* species or AG could present in the same turfgrass site. There was no relationship between the geographic origin of the isolates and the clusters formed.

Introduction

Isolates of *Rhizoctonia* belong to a large and diverse group of soil borne fungi causing disease to many plant species producing fruits, vegetables, tubers and cereals (2). *R. solani* alone has been recorded to be pathogenic to at least 150 species (3). Multiple *Rhizoctonia* species have been reported to infect turfgrasses and these infections are collectively known as *Rhizoctonia* blight. Both warm-season and cool-season turfgrasses are susceptible to *Rhizoctonia* blight with the warm humid and warm tropical climatic zones highly favoring the disease (7). In the southern and transition zones of the United States, *Rhizoctonia* blight poses a major threat to successful growth and maintenance of several important turfgrass species. Tall fescue (*Festuca arundinacea* Schreb.), St. Augustinegrass [*Stenotaphrum secundatum* (Walter) Kuntze], zoysiagrasses (*Zoysia* spp.) and creeping bentgrass (*Agrostis stolonifera* L.) are a few such susceptible turfgrasses (7). Studies on *Rhizoctonia* diseases have revealed four species infecting turfgrasses. The most common *Rhizoctonia* species, *R. solani* Kühn (teleomorph: *Thanatephorus cucumeris* (Frank) Donk) is responsible for causing brown patch on cool season grasses and large patch on warm season grasses. Both *R. zeae* Voorhees (teleomorph: *Waitea circinata* var. *zeae* Warcup and Talbot) and *R. oryzae* Ryker and Gooch (teleomorph: *W. circinata* var. *oryzae* Warcup and Talbot) are responsible for leaf and sheath spot disease. The *Rhizoctonia* incited yellow patch caused by *R. cerealis* Van der Hoeven (teleomorph: *Ceratobasidium cereale* Murray and Burpee) is thought to be dominant on annual bluegrass (*Poa annua*) in the west, Midwest and Northeastern regions of the United States (14; 37). The teleomorph *Waitea circinata* var. *circinata* causing brown ring patch is the latest addition to *Rhizoctonia* diseases on turfgrasses. Brown ring patch was first reported from Washington State in 2003 and initial symptoms appeared similar to yellow patch (14). All above species have multinucleate cells except for *R. cerealis*, which is a binucleate species. Unlike other *Rhizoctonia* species *R. cerealis* favors cool and wet weather for disease initiation. There are numerous reports of other binucleate *Rhizoctonia*-like fungi (BNR) being isolated from turfgrass swards or soils (6; 7; 17). These differ from *R. cerealis* in morphological or physiological characters and anastomosis reactions. Pathogenicity tests conducted on a few occasions have revealed BNR to be non-pathogenic or weakly virulent on turfgrasses (6). Though disease symptoms of turfgrasses vary depending on the *Rhizoctonia* species, many other factors also may influence symptom development. The turfgrass species, mowing height, soil conditions and environmental conditions are such

elements (37). In general, affected turfgrasses show circular areas of blighted brown color leaves. Therefore reliance on field symptoms to identify *Rhizoctonia* causal agents can be misleading. Microscopically, in the asexual state all *Rhizoctonia* species look more or less similar, i.e. nonsporulating mycelia with 90 degree branches having dolipore septa (37). However, the colony morphology on potato dextrose agar (PDA) growth media could vary greatly for different *Rhizoctonia* species. For instance, *R. solani* produces brown to dark brown sclerotia while *Waitea* species have orange to salmon pink sclerotia (7; 37). Even with this macroscopic distinction in culture, distinguishing *Rhizoctonia* strains and species may often require additional morphological and physiological characteristics for proper identification (7).

The classical method of grouping isolates of *Rhizoctonia* is based on anastomosis with tester strains. Anastomosis is the hyphal fusion reactions of vegetatively compatible isolates. Isolates within an anastomosis group (AG) are genetically closer to one another than isolates of a different AG. Therefore AGs also represent genetic isolation (8; 32). The most wide spread pathogen, *R. solani*, is a species complex having 14 AGs (25). Previous research has reported six AGs infecting turfgrasses. Burpee and Martin (1992) in their review on *Rhizoctonia*, describe AG 1, AG 2-2, AG 4 and AG 5 to be associated with turfgrasses (7). Zhang and Dernoeden give references to six AGs isolated from turfgrasses (46). They include AG 1, AG 2-2, AG 3, AG 4, AG 5 and AG 6. Other *Rhizoctonia* species associated with turfgrasses have only one AG. For instance isolates of binucleate *R. cerealis* infecting turfgrasses belong to AG-D. Though anastomosis grouping has been used widely, sometimes anastomosis reactions are difficult to interpret and may take excessive amounts of time when grouping many isolates. Some isolates which are known as bridging isolates (BI) can anastomose with more than one AG leading to further confusion. Further, isolates within an AG may show different types of hyphal fusion reactions (non-perfect fusions) making it difficult to determine the proper anastomosis group (8). It is also important to note that isolates of different subgroups within an AG are not distinguishable on the basis of anastomosis reactions since they anastomose each other (8). Under these circumstances AGs themselves may not necessarily give information on the genetic variation and taxonomic relationships between them (8; 24). This has led to the necessity to develop additional identification methods which could supplement anastomosis reactions.

Since the mid 1990's molecular techniques based on polymerase chain reaction (PCR) have been largely used to determine genetic diversity and taxonomic classification of fungal

species (38). Many published research papers on *Rhizoctonia* species have utilized primers specific to ribosomal DNA internal transcribed spacer (rDNA-ITS) regions for studying genetic diversity between and within anastomosis groups (3; 15; 16; 21; 22; 33). In eukaryotic ribosomal genes, ITS regions (ITS 1 and ITS 2) are flanked by genes for the 18s and 28s subunits (45). The coding region for ribosomal gene 5.8s is within the ITS region. These ribosomal genes as well as ITS regions are found in multiples copies in tandem within the fungal genome. While the 5.8s subunit is completely conserved, both ITS1 and ITS2 regions show high variability (3; 21). The sequence polymorphism of the ITS regions have been used to construct phylogenetic trees by grouping of genetically similar isolates of *Rhizoctonia* together. (9; 15; 21). Analysis of the ITS region has the capacity to group isolates of *Rhizoctonia* into their AG subgroup level. Kuninaga et al. examined ITS regions of rDNA in 45 isolates (from different host species) representing 11 AGs and 11 AG subgroups of *R. solani* (21). They found sequence homology of ITS regions for isolates of the same subgroups was above 96%. Isolates of different subgroups within an AG had sequence similarity of 66-100%. Isolates of different AGs showed 55-96% sequence similarity. Among the isolates analyzed, the sequence of ITS1 was more variable than ITS2 region.

Various DNA fingerprinting methods based on PCR have been used for identification and classification of the *Rhizoctonia* species complex (25; 35). The universally primed PCR (UP-PCR) is a PCR fingerprinting method very similar to the random amplified polymorphic DNA (RAPD) technique (5). This technique can be used to generate multiple DNA fragments from an organism without prior knowledge of DNA sequences. The main difference between UP-PCR and RAPD is the length of the primers used. Universal primers (UP) are longer (15-21 nt) than conventional RAPD primers (typically 10 nucleotides in length) and designed to anneal under more stringent conditions (52– 60°C) ensuring higher reproducibility of banding profiles (26; 43). In contrast RAPD uses lower annealing temperatures (35-40°C) where the banding patterns have been shown to be less consistent even when the same experiment is repeated in the same laboratory. The universal primers (UP) were originally designed to target and multiply intergenic, phylogenetically less conserved regions of the genome (26). Depending on the fungus, some highly polymorphic amplified fragments may enable detection of infra-specific variation (5). This ability to distinguish among closely related strains is due to the reliable generation of a higher number of bands (60-100) than RAPD. Lübeck and Poulsen found considerable variation in UP banding profiles for different AGs and AG subgroups among

isolates of *R. solani* (25). Lübeck et al. used visual comparison of UP-PCR banding profiles to determine genetic variability of unknown *Trichoderma* strains (27). They compared banding profiles obtained from reference strains for species designation of unknown isolates (27).

Amplified fragment length polymorphism (AFLP) is a technique different from other fingerprinting methods. In this method, genomic DNA is digested with two restriction endonucleases and two double stranded oligonucleotide adapters are ligated to each fragment. These modified fragments are amplified by two primers recognizing the adapter sequences and adjacent restriction site using PCR (24; 44). The resulting banding patterns are highly reproducible and the proportion of the genome analyzed is larger than the RAPD technique (30). Though studies based on the AFLP technique have been scarcely employed in *Rhizoctonia*, genetic variation of isolates of many other fungal species have been ascertained using this method (1; 30). This method has a high potential to reveal cryptic genetic variations among closely related strains having similar morphological characters.

Only a few studies have been done to investigate genetic structure of *Rhizoctonia* species causing disease on cool-season turfgrasses in the transition zone. Martin and Lucas identified *R. solani, R. zeae* and BNR from diseased cool season turfgrasses in Raleigh, North Carolina area (31). They assigned isolates of *R. solani* to AGs 1, 2, 4 or 5. However, Martin and Lucas (31) did not further identify these isolates into their AG subgroups. In a different study, Zhang and Dernoeden could identify only *R. solani* AG1-IA and AG 2-2IIIB from diseased leaf lesions of cool-season perennial ryegrass and creeping bentgrass (47). Their survey was mainly confined to University of Maryland Turfgrass Research Facility in Silver Spring, Maryland (MD). Both Martin and Lucas (31), and Zhang and Dernoeden (47) used anastomosis reactions with tester strains and colony morphology for pathogen identification. No peer reviewed reports have been published on systematic surveying of several geographic locations, and characterization of pathogens causing *Rhizoctonia* blight in Virginia (VA). However, brown patch related diseases are common in VA since this is a part of the transition zone. In 2009, Kammerer et al. published a first report of brown ring patch caused by *W. circinata* var. *circinata* in northern VA (18). Only a few previous studies had relied on molecular techniques for identification of *Rhizoctonia* species infecting cool-season grasses in the transition zone. Therefore, our study was carried out to investigate different *Rhizoctonia* species and subpopulations causing disease on turfgrasses in different geographies of VA and MD. One of the main goals of our study was to analyze and

compare highly variable isolates of *Rhizoctonia* using different molecular techniques. We used several molecular techniques since previous studies do not indicate which molecular technique is most suited to analyze a sample of *Rhizoctonia* isolates comprising different species and AGs.

We hypothesized that, i) *Rhizoctonia* isolates infecting turfgrasses belong to different species and genetically different subpopulations; ii) there is an established major pathogen for different geographies; iii) *Rhizoctonia* subpopulations represented by lawns and golf greens are different. To test these hypotheses, we surveyed different geographical locations of VA and MD and where possible sampled diseased leaves of turfgrasses from lawns and golf putting greens. The sampled *Rhizoctonia* isolates were analyzed using ITS ribotyping, UP-PCR and AFLP techniques. Thereafter, we assessed the compatibility of classical anastomosis grouping of *Rhizoctonia* isolates with the groups derived by the molecular techniques.

Materials and Methods

Sample collection and pathogen isolation

Isolates of *Rhizoctonia* were collected from diseased turfgrasses during summer months of 2007 through 2009. They were collected from lawns, golf course fairways and greens in VA and MD (Figure 2.1). Three sites of a selected general area were extensively sampled to isolate on average 10-30 fungal isolates per site. The only exception was Virginia Beach for which samples were available from one single site. The diseased grass pieces were rinsed in distilled water and placed in 2% water agar plates for isolating *Rhizoctonia* strains. *Rhizoctonia* like cultures were verified by observing under microscope and transferred to quarter strength potato dextrose agar (¼ x PDA) petri plates for obtaining pure cultures. Vials of sterile wheat seeds were inoculated with *Rhizoctonia* cultures for long term preservation. Sterile wheat seeds were prepared by initially soaking them in water overnight, thereafter draining excess water and autoclaving seeds twice at 121°C for 60 minutes in two consecutive days. Once *Rhizoctonia* strains had fully colonized the substrate, 15% glycerol was added to the vials, which were then stored at -75°C.

Figure 2.1 Geographic locations used to collect *Rhizoctonia* isolates in Maryland and Virginia.

Virginia Maryland

From the total collection of more than 400 *Rhizoctonia* isolates, a stratified random sample of 86 representing all geographies was used in this study (Table 2.1). The total *Rhizoctonia* collection was grouped into different geographic areas before sampling was done. The sample size of each location was proportionate to the total collection representing each area. *Waitea* species were first identified from the sample, using colony morphology on PDA plates. Isolates which did not belong to *Waitea* species were grouped into binucleates and multinucleates by staining with 4´, 6-diamidino-2-phenylindole (DAPI) and observing under a fluorescence microscope for number of nuclei present per fungal cell.

Anastomosis grouping

Where possible, isolates of *Rhizoctonia* were grouped into their respective AGs using tester strains of *R. solani, Waitea circinata* varieties and binucleate *R. cerealis*. Past research work had identified six AGs (AG 1 through AG 6) from turfgrasses for *R. solani* (7; 46). Isolate pairing was done on large petri plates having a thin layer of 1.5% water agar. A mycelial plug of the tester strain was placed at the center and paired with 3 plugs of the field strain placed near the edge of the plate at equal distance from each other. An inverted phase contrast microscope was used to observe hyphal pairings. Hyphal fusion reactions observed with one field plug and the center tester strain was considered as one replicate. Therefore one plate consisted of 3 replicates of hyphal fusion reactions. Tester strains AG 1-1A, AG 1-IB, AG 2-2IIIB, AG 3, AG 4, AG 5 and AG 6 were paired with unknown multinucleate *R. solani* isolates to determine their AG. Grouping of unknown isolates into their respective AGs were done in accordance to the method described by Carling et al. (9) and Macnish and Carling (29). In their categories, C0 indicates no hyphal fusion; C1 represents contact of hyphal points with no cytoplasmic fusion; C2 indicates cell death soon after cell wall and cytoplasmic fusion; C3 involves perfect fusion with cell wall

Table 2.1 Geographic origin, host and anastomosis group of isolates used in this study

Isolate	Host	Origin	Species	AG/subgroup
UMTRC 114	Tall fescue	Beltsville, MD	Rs	AG 2-2IIIB
UMTRC 150	Tall fescue	Beltsville, MD	Rs	AG 2-2IIIB
UMTRC 262	Tall fescue	Beltsville, MD	Rs	AG 2-2IIIB
RS 6	CBG/ABG	Blacksburg, VA	Rs	AG 2-2IIIB
RS 13	CBG/ABG	Blacksburg, VA	Rs	AG 2-2IIIB
GC 20C	CBG/ABG	Blacksburg, VA	Rs	AG 2-2IIIB
GC 22C	CBG/ABG	Blacksburg, VA	Rs	AG 2-2IIIB
GC 32C	CBG/ABG	Blacksburg, VA	Rs	AG 2-2IIIB
BSF 69	Tall fescue	Richmond, VA	Rs	AG 2-2IIIB
BSF 50	Tall fescue	Richmond, VA	Rs	AG 2-2IIIB
BSF 42	Tall fescue	Richmond, VA	Rs	AG 2-2IIIB
BSF 90	Tall fescue	Richmond, VA	Rs	AG 2-2IIIB
BSF 207	Tall fescue	Richmond, VA	Rs	AG 2-2IIIB
BSF 209	Tall fescue	Richmond, VA	Rs	AG 2-2IIIB
SF 214	Tall fescue	Richmond, VA	Rs	AG 2-2IIIB
RSF 127	Tall fescue	Richmond, VA	Rs	AG 2-2IIIB
LB 312	Tall fescue	Leesburg, VA	Rs	AG 2-2IIIB
LB 317	Tall fescue	Leesburg, VA	Rs	AG 2-2IIIB
LB 325	Tall fescue	Leesburg, VA	Rs	AG 2-2IIIB
HDN 102	CBG/ABG	Herndon, VA	Rs	AG 2-2IIIB
HDN 208By	Tall fescue	Herndon, VA	Rs	AG 2-2IIIB
HDN 225	Tall fescue	Herndon, VA	Rs	AG 2-2IIIB
GCGC 118B	Tall fescue	Leesburg, VA	Rs	AG 2-2IIIB
GCGC 303	Tall fescue	Leesburg, VA	Rs	AG 2-2IIIB
GCGC 316	CBG/ABG	Leesburg, VA	Rs	AG 2-2IIIB
GCGC 319	Tall fescue	Leesburg, VA	Rs	AG 2-2IIIB
ANP 202B	Tall fescue	Annapolis, MD	Rs	AG 2-2IIIB
ANP 205A	Tall fescue	Annapolis, MD	Rs	AG 2-2IIIB
ANP 205B2	Tall fescue	Annapolis, MD	Rs	AG 2-2IIIB
ANP 309A	Tall fescue	Annapolis, MD	Rs	AG 2-2IIIB
BARC 26	Tall fescue	Beltsville, MD	Rs	AG 1-IB
BARC 02	Tall fescue	Beltsville, MD	Rs	AG 1-IB
MP 32	Tall fescue	Blacksburg, VA	Rs	AG 1-IB
MP 51	Tall fescue	Blacksburg, VA	Rs	AG 1-IB
MP 43	Tall fescue	Blacksburg, VA	Rs	AG 1-IB
MP 35	Tall fescue	Blacksburg, VA	Rs	AG 1-IB
LB 123	Tall fescue	Leesburg, VA	Rs	AG 1-IB
LB 124	Tall fescue	Leesburg, VA	Rs	AG 1-IB
LB 127	Tall fescue	Leesburg, VA	Rs	AG 1-IB
LB 234	Tall fescue	Leesburg, VA	Rs	AG 1-IB

Table 2.1 Continued

Isolate	Host	Origin	Species	AG/subgroup
PW 326	Tall fescue	Woodbridge, VA	Rs	AG 1-IB
PW 353	Tall fescue	Woodbridge, VA	Rs	AG 1-IB
HDN 111A	Tall fescue	Herndon, VA	Rs	AG 1-IB
HDN 122A	Tall fescue	Herndon, VA	Rs	AG 1-IB
HDN 302	Tall fescue	Herndon, VA	Rs	AG 1-IB
GCGC 217	Tall fescue	Leesburg, VA	Rs	AG 1-IB
ANP 301B	Tall fescue	Annapolis, MD	Rs	AG 1-IB
ANP 306B	Tall fescue	Annapolis, MD	Rs	AG 1-IB
LB 204	Tall fescue	Leesburg, VA	Rs	AG 5
UMTRC 122	Tall fescue	Beltsville, MD	BNR	unknown
BARC 17	Tall fescue	Beltsville, MD	BNR	unknown
SF 224	Tall fescue	Richmond, VA	BNR	unknown
LB 226	Tall fescue	Leesburg, VA	BNR	unknown
PW 154	Tall fescue	Woodbridge, VA	BNR	unknown
PW 205	Tall fescue	Woodbridge, VA	BNR	unknown
PW 216	Tall fescue	Woodbridge, VA	BNR	unknown
PW 249	Tall fescue	Woodbridge, VA	BNR	unknown
PW 341	Tall fescue	Woodbridge, VA	BNR	unknown
HDN 209	Tall fescue	Herndon, VA	BNR	unknown
HDN 221	Tall fescue	Herndon, VA	BNR	unknown
HDN 324A	CBG/ABG	Herndon, VA	BNR	unknown
HDN 325B	CBG/ABG	Herndon, VA	BNR	unknown
GCGC 202A	Tall fescue	Leesburg, VA	BNR	unknown
GCGC 214A	Tall fescue	Leesburg, VA	BNR	unknown
ANP 107	Tall fescue	Annapolis, MD	BNR	unknown
BARC 05	Tall fescue	Beltsville, MD	Wcz	WAG-Z
LB 319	Tall fescue	Leesburg, VA	Wcz	WAG-Z
LB 228	Tall fescue	Leesburg, VA	Wcz	WAG-Z
PW 220	Tall fescue	Woodbridge, VA	Wcz	WAG-Z
PW 119	Tall fescue	Woodbridge, VA	Wcz	WAG-Z
HDN 115A	Tall fescue	Herndon, VA	Wcz	WAG-Z
HDN 211	Tall fescue	Herndon, VA	Wcz	WAG-Z
GCGC 116	Tall fescue	Leesburg, VA	Wcz	WAG-Z
VABCH 08	Tall fescue	Virginia Beach, VA	Wcz	WAG-Z
VABCH 10	Tall fescue	Virginia Beach, VA	Wcz	WAG-Z
UMTRC 159	Tall fescue	Beltsville, MD	Wcz	WAG-Z
GCGC 220	CBG/ABG	Leesburg, VA	UWC	WAG
ANP 109B	Tall fescue	Annapolis, MD	UWC	WAG
ANP 304	Tall fescue	Annapolis, MD	UWC	WAG
HDN 222A	Tall fescue	Herndon, VA	UWC	WAG
UMTRC 228	Tall fescue	Beltsville, MD	UWC	WAG

Table 2.1 Continued

Isolate	Host	Origin	Species	AG/subgroup
RSF 13	Tall fescue	Richmond, VA	UWC	WAG
TRC 211	CBG/ABG	Blacksburg, VA	Wcc	WAG
TRC 216	CBG/ABG	Blacksburg, VA	Wcc	WAG
TRC 202	CBG/ABG	Blacksburg, VA	Wcc	WAG
RZ 08	CBG/ABG	Blacksburg, VA	Wcc	WAG

ABG = annual bluegrass; CBG = creeping bentgrass
Rs = *R. solani*; BNR = binucleate *Rhizoctonia*-like fungi; Wcz = W. *circinata* var. *zeae;* Wcc = *W. circinata* var. *circinata*; UWC = unidentified *W. circinata* species

and cytoplasmic fusion with no cell death. Observation of at least five perfect anastomosis points per replicate permits the allocation of an unknown isolate into the AG of the tester strain. Isolates with morphological characteristics of *Waitea* species were anastomosed with tester stains *W. circinata* var. *zeae, W. circinata* var. *oryzae* and *W. circinata* var. *circinata.* Isolates of unknown binucleate *Rhizoctonia*-like fungi (BNR) were paired with *R. cerealis* (AG-D).

DNA extraction

Three mycelial plugs from each fungal isolate were cultured in 1000 ml Erlenmeyer flasks containing 100 ml of potato dextrose broth (Difco Laboratories, Detroit, MI, USA). The cultures were incubated at room temperature for 4 days. The mycelial mats were harvested onto a muslin cloth, rinsed thoroughly with distilled water and excess water was removed by pressing between several blotting papers. Thereafter 0.3 g of each fungal mat was lyophilized in liquid nitrogen, ground thoroughly and DNA extracted using the protocol given by the Qiagen DNeasy plant mini kit (Qiagen Inc, Valencia, CA).

rDNA-ITS region amplification

The ITS region of fungal DNA was amplified with the primer set of ITS1 (5´-TCCGTAGGTGAACCTGCGG -3´) and ITS4 (5´- TCCTCCGCTTATTGATATGC -3´). The reaction mixture of 25 µl contained 1 x standard reaction buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$) (New England Biolabs, Ipswich, MA, USA), 0.2 µM of each primer, 0.2 mM of 4dNTP, 1 U of Taq polymerase (New England Biolabs) and 30-50 ng of genomic DNA. PCR

was carried out by MJ Research PTC-200 thermocycler (Global Medical Instrumentation, Ramsey, MN, USA) using the following conditions: an initial denaturation at 94°C for 2 min, followed by 35 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 10 minutes. Thereafter the reaction was stopped by reducing the temperature to 4°C and PCR products were stored at -20°C. Aliquots of 5 µl of amplified products were electrophoresed on 1% (w/v) agarose gel in tris-borate-EDTA (TBE) buffer, stained with ethidium bromide and visualized under UV light for DNA fragments.

ITS sequencing and data analysis

The PCR products of the ITS region were purified using QuickStep™2 PCR Purification Kit (Edge Bio Systems, Gaithersburg, MD, USA) according to manufacturer's instructions. Purified ITS templates having a DNA concentration of 5-25 ng were amplified with an Applied Biosystems BigDye 3.1 sequencing kit. The reaction mixture of 10 µl contained 1 µl BigDye, 1.8 µl of 5 x BigDye buffer, 2.5 µl of 1 µM primer (either ITS1 or ITS4) and distilled water. The PCR was performed in a thermocycler (MJ Research PTC-200) with initial denaturation at 94°C for 2 min and thereafter 35 cycles of 94°C for 15 s, 50°C for 15 s and 60°C for 4 min. The amplified products were sequenced by an ABI Prism 3730 (Applied Biosystems, Foster City, CA, USA). The forward and reverse sequences were assembled using Lasergene SeqMan suite (DNASTAR, Madison, WI, USA) and consensus sequences were derived for each sample. The sequence data of all isolates were aligned by MEGA 4 software program using the Clustal W algorithm (40). The existing sequences of relevant AGs of *Rhizoctonia* strains from GenBank (http://www.ncbi.nlm.nih.gov) were also used in the alignment. The alignment of all sequences was checked visually and obvious errors were adjusted. A phylogenetic tree was created from distance matrix values by the neighbor- joining method using MEGA 4 software. Kimura's two parameter model was used to determine the distances of the aligned ITS sequences (20). The clades of the tree were midpoint rooted. Branch support of the tree was calculated by bootstrap analysis based on 500 replicate heuristic searches.

UP-PCR product generation

A UP-PCR reaction mixture of 25 µl containing 1 x standard Taq polymerase reaction buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl) (New England Biolabs, Ipswich, MA,

USA), 0.2 mM dNTPs, 0.4 µM of primer, 30-50 ng of DNA template, and 1 unit of Taq polymerase (New England Biolabs) was used for each reaction. Four UP primers were tested initially using 10 isolates of *Rhizoctonia* to determine which primers gave the best banding profile. The primers tested were as follows: L45 (5´-GTAAAACGACGGCCAGT-3´), L15/AS19 (5´-GAGGGTGGCGGCTAG-3´), AS15 (5´-GGCTAAGCGGTCGTTAC-3´), and L21(5´-GGATCCGAGGGTGGCGGTTCT-3´) (12; 26). The UP-PCR was performed in a PTC-200 thermocycler (Global Medical Instrumentation, Ramsey, MN, USA). The initial denaturation was done at 94°C for 2 min, followed by 35 cycles with DNA denaturation at 94°C for 1 min, primer annealing at 52°C for 1 min and primer extension at 72°C for 1 min. The final extension step was performed at 72°C for 10 min. To determine DNA banding profile, 5 µl of PCR products of each isolate were electrophoresed in 1.7% agarose gel for one hour at 100 V. The gel was stained with ethidium bromide and visualized using the AlphaImager 3400 (Alpha Innotech Corporation, San Leandro, CA). All four tested primers gave satisfactory banding profiles. Out of four UP primers, L21 and L15/AS19 were selected to analyze the *Rhizoctonia* isolates (see Table 2.1). These primers were tagged with fluorescent dye 6-FAM in order to digitally analyze PCR products using an ABI Prism 3730 capillary electrophoresis system. Five tester strains (AG 2-2IIIB, AG 5, Wcc, Wcz and Wco) were also included in the analysis.

Analysis of UP-PCR data

The PCR products were prepared for analysis by mixing 0.75 µl of each sample with 10 µl of Hi-Di Formamide (Applied Biosystems) and 0.4 µl of internal marker GeneScan™ 1200 LIZ® (Applied Biosystems). These samples were denatured for 5 min at 94°C, chilled immediately for several minutes on ice and loaded on genetic analyzer ABI Prism 3730 (Applied Biosystems, Foster City, CA) for fragment analysis. The GeneScan™ 1200 LIZ® has the ability to size fragments between 20 to 1200 bp. Bands smaller than 50 bases were not scored to avoid primer sequences. Fragment analysis and phenotypic clustering was done as explained under AFLP analysis.

Table 2.2 Amplified fragment length polymorphism (AFLP) adapter, pre-amplification primer and selective amplification primer sequences used for analysis of the sample isolates

EA1	5´-CTCGTAGACTGCGTACC-3´
EA2	3´-CATCTGACGCATGGTTAA-5´
MA1	5´-GACGATGAGTCCTGAG-3´
MA2	3´-TACTCAGGACTCAT-5-3´
*Eco*RI primer + *A*	5´-GACTGCGTACCAATTC*A*-3´
*Mse*I primer + *C*	5´-GATGAGTCCTGAGTAA*C*-3´
*Eco*RI primer + *AAA*	5´-ACTGCGTACCAATTC*AAA*-3´
*Mse*I primer + *CTA*	5´-GATGAGTCCTGAGTAA*CTA*-3´
*Eco*RI primer + *AAC*	5´-ACTGCGTACCAATTC*AAC*-3´
*Mse*I primer + *CTA*	5´-GATGAGTCCTGAGTAA*CAC*-3´
*Eco*RI primer + *AGT*	5´-ACTGCGTACCAATTC*AGT*-3´
*Mse*I primer + *CTG*	5´-GATGAGTCCTGAGTAA*CGT*-3´

AFLP analysis

The AFLP analysis was based on the method described by Ceresini et al. and Vos et al. with minor modifications (10; 44). Approximately 100 ng of genomic DNA was digested with *Eco*RI (New England BioLabs, Beverly, MA, USA) and *Mse*I (New England BioLabs). Thereafter digested products were ligated with *Eco*RI double stranded (ds) adapter (EA1 and EA2) and *Mse*I ds adapter (MA1 and MA2) sequences to respective *Eco*RI and *Mse*I sites (See Table 2.2 for sequences). Both digestion and ligation reactions were done in one step by preparing a reaction mixture of 20 µl having 2 U of each restriction enzyme, 1.2 U of T4 DNA ligase (New England BioLabs) and 100 ng of DNA template. The reaction mixture also included 1 x *Eco*RI reaction buffer (50 mM NaCl, 100 mM Tris-HCl, 10 mM $MgCl_2$, 0.025% Triton X-100), 1 x *Mse*I reaction buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT), 1 x T4 ligase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP), 2µg of bovine serum albumin, 0.1 µM of *Eco*RI adapter and 1 µM of *Mse*I adapter. The reaction mixture was incubated overnight to complete digestion and ligation reactions. After incubation, the reaction mixture was diluted ten-fold by adding sterile TE (Tris-EDTA) buffer and stored at -20°C to use later. The first amplification (pre-amplification) was carried out with *Eco*RI and *Mse*I primers

with one selective nucleotide (Table 2.2). They were *Eco*RI primer + A and *Mse*I primer + C. The sample of 25 µl included 5µl of DNA template from previous step, 0.5 µM each of *Eco*RI and *Mse*I primers, 1 x Taq polymerase reaction buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$), 0.2 mM 4dNTP and 1 U of New England BioLabs Taq polymerase. The PCR was performed in a MJ Research PTC-200 thermocycler with initial denaturation at 94°C for 2 min followed by 20 cycles of 30 s at 94°C, 1 min at 56°C and 1 min at 72°C. The PCR products were diluted ten-fold with TE buffer and used as the template DNA for the selective amplification step. For selective amplification three primer pairs consisting of *Eco*RI and *Mse*I with 3 selective nucleotides each were used (see Table 2.2). All three *EcoRI* primers (Table 2.2) were end labeled with fluorescence dye 6-FAM at the 5´ end. *Eco*RI primers were synthesized by Eurofins MWG Operon (Huntsville, AL, USA). *Mse*I primers were synthesized by IDT (Integrated DNA Technologies, Coralville, IA, USA). Each selective PCR mixture of 20 µl included 4 µl of secondary DNA template, 0.5 µM each of *Eco*RI and *Mse*I primers, 1 x standard Taq polymerase reaction buffer, 0.2 mM 4dNTP and 1 U of New England BioLab Taq polymerase. The PCR reaction was performed for 36 cycles with the following cycle profile. Cycle 1 with 30 s DNA denaturation step at 94°C, 30 s annealing step at 65°C, and 1 min extension step at 72°C. Cycle 2-12 as in cycle 1, but with a progressive drop in the annealing temperature of 0.7°C in each cycle. Cycles 13-36 included 30 s at 94°C, 30 s at 56°C and 1 min at 72°C. A final extension step of 5 min at 72°C completed the reaction. AFLP banding profiles were determined by electrophoresing 5µl of PCR samples in 1.7% agarose gel for one hour at 100 V and visualizing ethidium bromide stained gels under UV light.

AFLP data analysis

Following PCR amplification and band visualization, 0.5 µl of each of PCR samples were mixed with 9 µl Hi-Di formamide (Applied Biosystems, Warrington, UK) and 0.5 µl of GeneScan™ 500LIZ® internal marker (Applied Biosystems, Foster City, CA, USA). The mixtures were denatured at 95°C for 5 min and placed on ice for few minutes. Thereafter samples were loaded onto ABI model 3730 (Applied Biosystems Inc.) and analyzed for DNA banding profiles. The 500LIZ® internal maker enabled the automated data analysis for the capillary electrophoresis and has a DNA fragment sizing range of 35-500 bp. The GeneMapper software V3.7 (Applied Biosystems) was used to extract and analyze row data files obtained

from the ABI 3730. AFLP profiles are binomially scored as zeros and ones by this software. A zero represents no fragment and a one indicates presence of a band of a particular base length. AFLP band polymorphism was treated as a phenotype since comigrating fragments may not necessarily have a common descent. The scoring table of zeros and ones was imported to MEGA 4 software for constructing dendrogram based on neighbor-joining method (34).

Results

Anastomosis grouping

Each unknown *R. solani* isolate anastomosed only with testers belonging to one AG. Anastomosis reactions made it possible to group *R. solani* isolates into three AGs. They were AG 2-2, AG 1 and AG 5. C3 reactions were observed from both AG 1-1A and AG 1-IB testers when paired with suspected isolates of AG 1. The morphology of sclerotia could distinguish *R. solani* AG 1 from AG 2-2. Isolates of AG 1 produced several irregular, large aggregated sclerotia (2 - >5 mm) in the center and periphery of ¼ x PDA petri plates. They were dark brown and over time produced phenolic exudates. Investigated isolates of AG 2-2 produced numerous, round to irregular, small (<1 mm), dark brown, scattered sclerotia on ¼ x PDA plates. There were 30 isolates of AG 2-2; 18 isolates of AG 1 and one isolate of AG 5. Subsequent molecular identification indicated *R. solani* AG 1 and AG 2-2 isolates belonged to subgroups IB and IIIB respectively.

Isolates of BNR did not anastomose with any tester strains including binucleate AG-D (*R. cerealis*). Most of the BNR isolates did not produce sclerotia on ¼ x PDA. However, a few cultures produced white to buff, medium to large (1–5 mm), and irregular sclerotia after incubation for three weeks at room temperature. Morphology of sclerotia could not be used to identify isolates of BNR.

Unknown isolates having characters of *Waitea* species (white to orange to pink color, spherical to irregular sclerotia on ¼ x PDA plates) anastomosed with all three *W. circinata* testers but did not pair with testers of *R. solani* or *R. cerealis*. Out of 86 isolates studied, there were 16 isolates of BNR and 21 isolates of *W. circinata*. Subsequent ITS sequence analysis revealed three groups of *W. circinata* including 11 isolates of *W. circinata* var. *zeae* (Wcz), four isolates of *W. circinata* var. *circinata* (Wcc) and six unknown isolates of *W. circinata* (UWC).

Diseased leaf samples from creeping bentgrass\annual bluegrass (CBG\ABG) (n=14) putting greens resulted in seven isolates of *R. solani* AG 2-2IIIB, four isolates of Wcc, two isolates of BNR and a single isolate of UWC . Nearly 32% (23/86) of all isolates from tall fescue were AG 2-2IIIB. All isolates of AG 1-IB (n=18) and Wcz (n=11) came from tall fescue. A high number of BNR (n=14) was also isolated from tall fescue. Table 2.3 summarizes these results.

Table 2.3 The species and anastomosis group designation of the *Rhizoctonia* sample and their host species

Rhizoctonia group	Number of isolates from Tall fescue	Number of isolates from CBG\ABG
R. solani AG 1-IB	18	-
AG 2- 2IIIB	23	7
AG 5	1	-
W. circinata var. *zeae*	11	-
var. *circinata*	-	4
Unidentified *W. circinata*	5	1
Binucleate *Rhizoctonia*-like fungi	14	2
Total	72	14

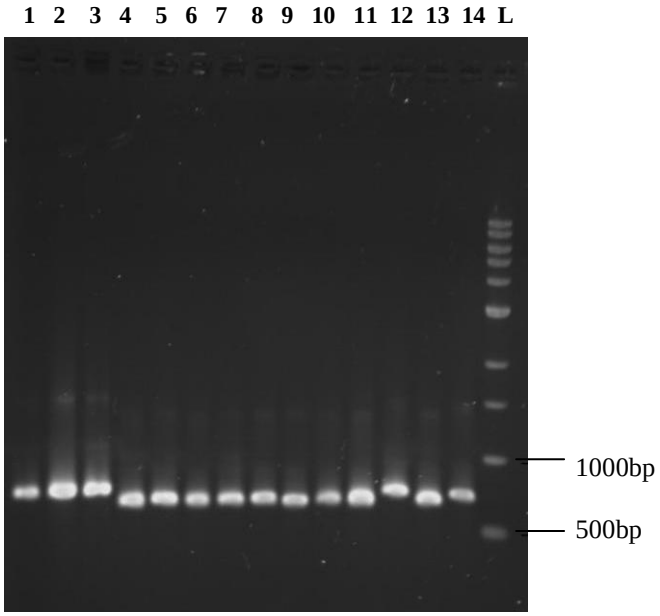
ABG = annual bluegrass; CBG = creeping bentgrass

ITS analysis

The amplified ITS region for the investigated isolates of *Rhizoctonia* produced a DNA fragment between 550 and 750 bp (Figure 2.2). The ITS sequence matrix had 820 nucleotide aligned positions including gaps. Out of this, 306 positions were removed from the analysis because they could not be unambiguously aligned. Therefore aligned sequences of studied isolates consisted of 514 nucleotide positions including gaps. Phylogenetic analysis of ITS sequences revealed well supported clades for *R. solani*, *W. circinata*, and BNR isolates (Figure 2.3). *R. solani* isolates grouped into three clusters and they corresponded well with previously identified AGs or AG subgroups (i.e. AG 1-IB, AG 2-2IIIB and AG 5) with bootstrap support of >99% (Figure 2.3, 2.4 and 2.5). All isolates identified as AG 1 by anastomosis grouped together with a confirmed AG 1-IB ITS sequence obtained from GenBank (accession no. AY152695). AG 1-IA accessions AB122133 and AB122134 clustered separately from field isolates (Figure

2.3). A confirmed AG 2-2IIIB ITS sequence from GenBank (AF354116) clustered with isolates which paired with testers of AG 2-2IIIB (Figure 2.4). Similarly AG 5 accessions AF153778 and

Figure 2.2 PCR products of internal transcribed spacer (ITS) region of 14 *Rhizoctonia* isolates (lanes 1 through 14) amplified with ITS1 and ITS4 primers. Lane L is a 1 kb mass ladder.



AF354113 grouped with isolate LB 204 which was previously identified as the sole AG 5 isolate in our study (Figure 2.3).

ITS sequences of *W. circinata* grouped into four clades (Figure 2.6). Two clades corresponded with *W. circinata* var. *zeae* (Wcz) and *W. circinata* var. *circinata* (Wcc). Corresponding GenBank accessions clustered together with these isolates. The third clade included six unidentified *W. circinata* (UWC) isolates. Out of those, five isolates grouped closely with *W. circinata* var. *agrostis* sequences (AB213572, AB213578) deposited in GenBank, while one isolate (RSF1-3) grouped closely with a new *W. circinata* variety (proposed name *W. circinata* var. *prodigus*) isolated from Florida golf courses (accessions HM597147, HM597146, HM597145) by Kammerer et al. (19)). *W. circinata* var. *oryzae* accessions (AB213589, AJ000195 and AB213588) consisted of the fourth clade. Two isolates (BARC 05 and UM 159) with characteristics of *W. circinata* var. *zeae* grouped outside all *W. circinata* clades described above.

Out of 16 BNR isolates, 15 grouped together while one isolate (BARC 17) grouped separately. Sequence similarity of this isolate was closer to AG-D (GenBank accessions AB198693 and AB198714) than other BNR isolates. All accession numbers of ITS sequences obtained from GenBank are indicated in the phylogenetic tree. There was no relationship between geographic origin and clades of the phylogenetic tree since *Rhizoctonia* isolates within a clade represent most of the sampling areas. Isolates of Wcc were found only from Blacksburg, VA while isolates of Wcz were not identified from Blacksburg or Richmond, VA.

ITS sequence similarity within a single clade was very high except for AG 1-IB (Table 2.4). Sequence similarity of *R. solani* isolates of AG 1-IB varied from 90.1-100%. All other clades including BNR and *W. circinata* had a sequence similarity greater than 96.5% (Table 2.4). In general, sequence similarity between unrelated groups was low and varied widely. Average percent genetic similarity between BNR and AGs of *R. solani* was equal to or greater than 82.6% (Table 2.5). All *W. circinata* groups (Wcc, Wcz and UWC) showed low genetic similarity with BNR and *R. solani* clades. Between these groups the average sequence similarity varied from 60.2% (between BNR and Wcc/UWC) to 54.7% (between AG 1-IB and Wcc) (Table 2.5). However, average sequence similarity between *W. circinata* groups were comparatively higher (93.5-94.4%) than AGs of *R. solani* (88.7-92.9%), though sampling within AG 5 was limited to one isolate.

Table 2.4 Pairwise percent similarity ranges of rDNA-ITS sequences for isolates within AGs and subgroups of *R. solani*, *W. circinata* and binucleate *Rhizoctonia*-like fungi (BNR) (see Figures 2.3 and 2.6)

	AG 5*	AG 2IIIB	AG 1-IB	BNR**	Wcz***	UWC	Wcc
% similarity	n/a	96.5-100	90.1-100	98.7-100	97-99.5	98.6-100	99.7-100

Wcz = W. *circinata* var. *zeae*; Wcc = *W. circinata* var. *circinata*; UWC = unidentified *W. circinata* species

* Investigated sample had only one isolate

** BARC 17 not included

*** BARC 5 and UM 159 not included

UP-PCR analysis

UP-PCR analysis included all the isolates in Table 2.1 except for isolate BNR 209. Isolate BNR 209 had to be removed from the analysis since it gave high background noise or numerous stutter peaks which were difficult to separate from actual peaks. Repeated attempts failed to produce acceptable results for this isolate. The digital analysis of UP fragments by capillary electrophoresis could resolve and capture bands up to one base pair difference. Primer L21 revealed a total of 373 markers (bands) for the total sample set including tester strains.

Table 2.5 Average percent homology of rDNA-ITS sequences for clades of AGs and subgroups of *R. solani, W. circinata* and binucleate *Rhizoctonia*-like fungi (BNR)

	AG 5	AG 2- 2IIIB	AG 1-IB	BNR	Wcz	UWC	Wcc
AG 5							
AG 2-2IIIB	92.9						
AG 1-IB	89.2	88.7					
BNR	85.7	85	82.6				
Wcz	57.7	56	56.6	59.7			
UWC	57.4	55.4	56.5	60.2	94.4		
Wcc	57.8	56.1	54.7	60.2	93.5	94.2	

Wcz = W. *circinata* var. *zeae*; Wcc = *W. circinata* var. *circinata*; UWC = unidentified *W. circinata* species

Primer L15/AS19 gave 380 fragments. Analysis of markers generated only from L21 clustered most of the isolates correctly into their infraspecies level which corresponded well with their AGs and subgroups. However, bootstrap values of branches were low (less than 46% for all major clusters) indicating less reliability. When L15/AS19 primer was combined with L21, the bootstrap values increased significantly (Figure 2.7). L15/AS19 primer alone did not resolve all isolates into their proper subgroups.

The dendrogram for the isolates gave seven clusters including three clades for isolates of *R. solani* and each of the *Waitea circinata* subgroups (Figure 2.7). Isolates of BNR clustered separately except for BARC17 and PW 249 (Figure 2.7). There was no association of geographic origin of the isolates and clusters. The AG of the isolates of *R. solani* and *W. circinata* corresponded strongly to the clusters formed in the dendrogram. Six isolates of *W. circinata* (UWC) grouped separately from known groups of *W. circinata* var. *zeae,* var. *circinata,* and var. oryzae (Wcz, Wcc and Wco). ITS analysis also grouped the same isolates together (Figure 2.6).

Figure 2.3 A neighbor-joining tree of investigated isolates of *Rhizoctonia* species clustered by analysis of rDNA-ITS sequences. Distances were calculated using Kimura two parameter. Bootstrap values greater than 50% with 500 replications are shown next to the branches. Clades having isolates of AG 2-2IIIB, AG 1-IB and *Waitea circinata* were collapsed to show the entire phylogenetic tree. The tree is midpoint rooted. The GenBank accessions used in the analysis are indicated by their accession number in the tree. *Rhizoctonia solani* anastomosis groups (AGs) AG 1-IA, -IB, 2-2IIIB and 5 are indicated in the tree. Isolates of *R. cerealis* are represented by the cluster AG-D. The clade for binucleate *Rhizoctonia*-like fungi is indicated by BNR.

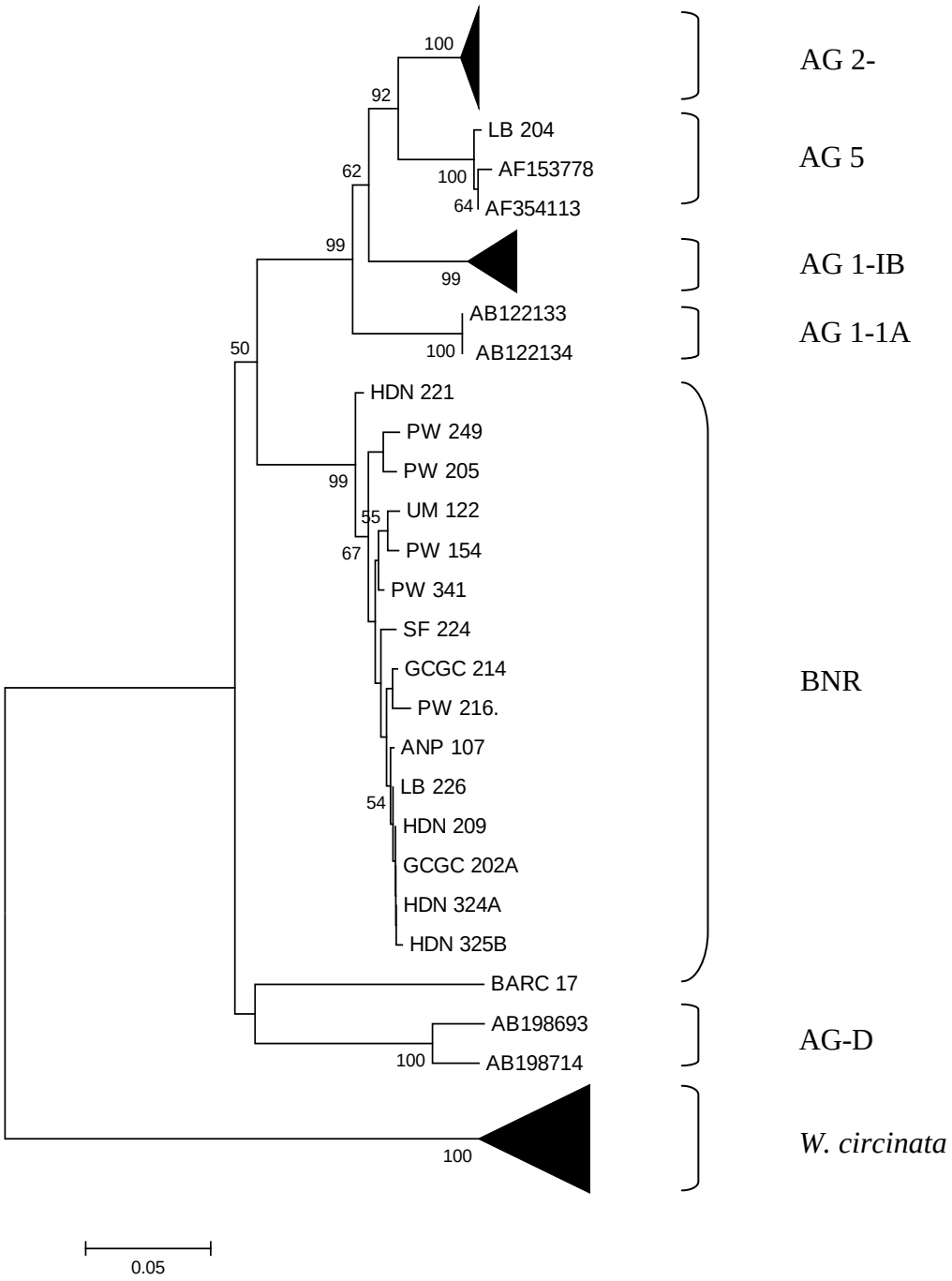


Figure 2.4 The detailed neighbor-joining sub-tree of collapsed AG 2-2IIIB clade indicated in Figure 2.3. AG 5 clade is also included in order to show bootstrap value of the sub-tree. Bootstrap values greater than 50% with 500 replications are shown at the beginning of the branch.

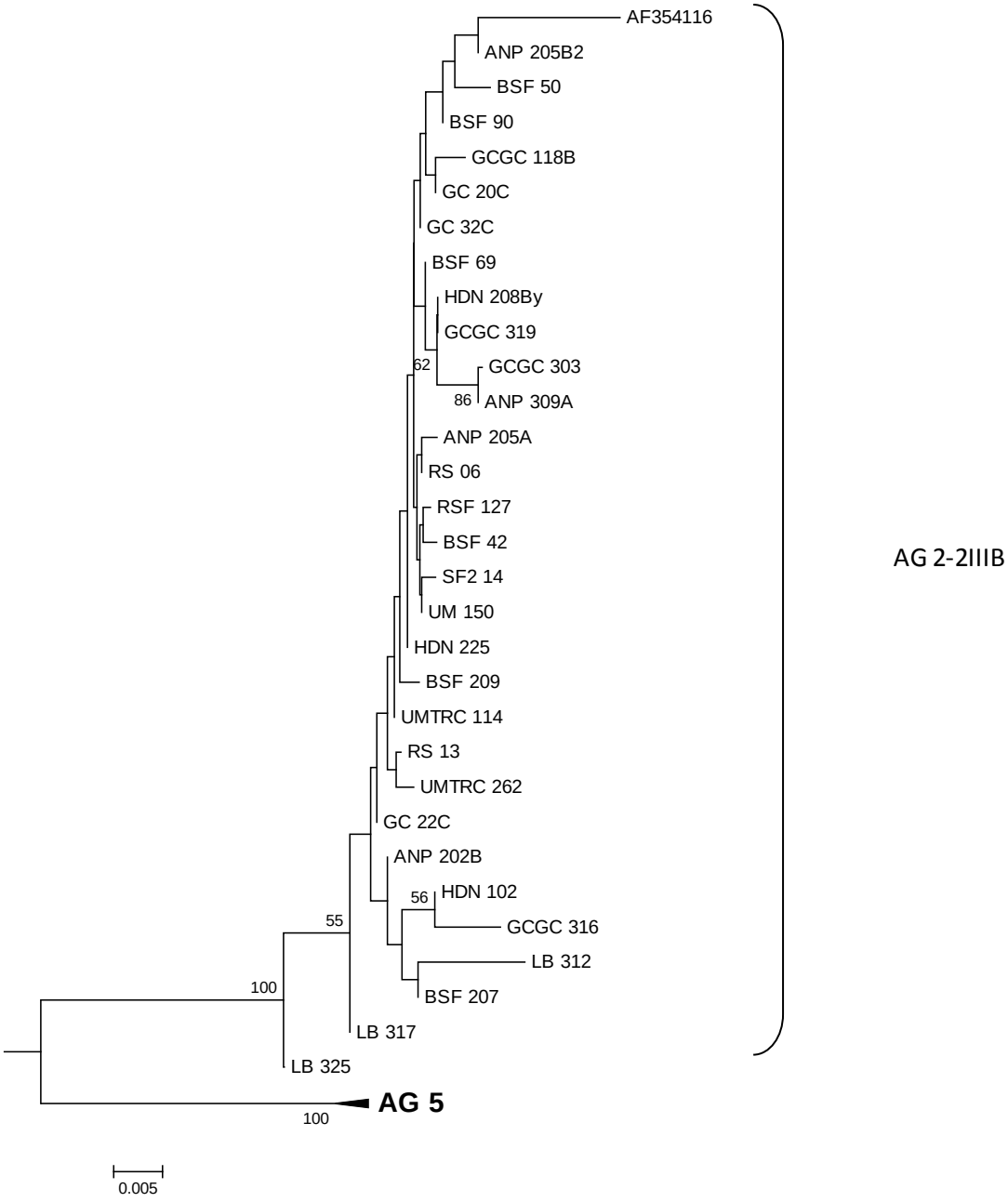


Figure 2.5 The neighbor joining sub-tree of collapsed AG 1-IB clade of Figure 2.3. AG 5 and AG 2-2IIIB clades are also included in order to show bootstrap value of the sub-tree. Bootstrap values greater than 50% with 500 replications are shown next to the branches.

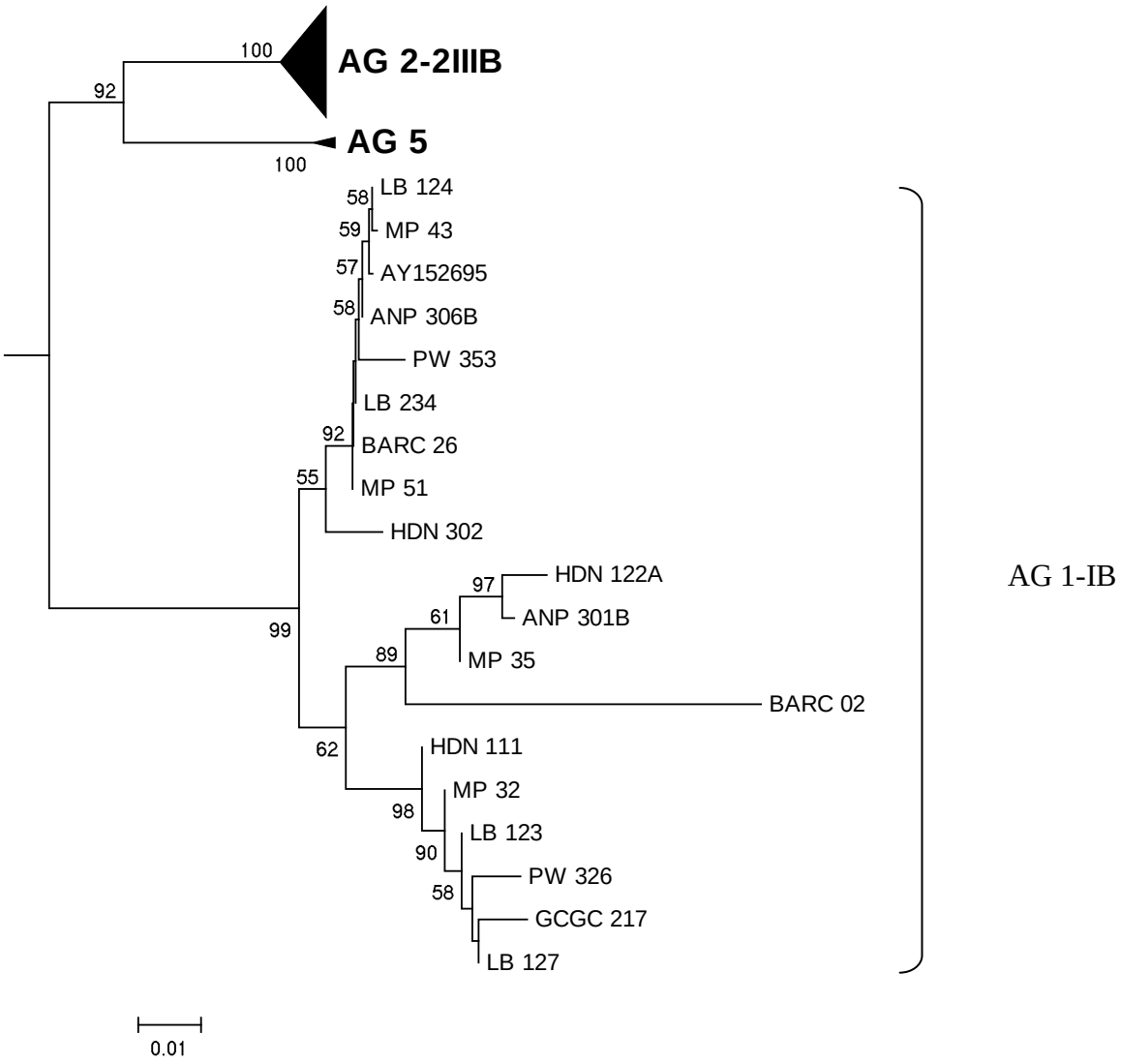
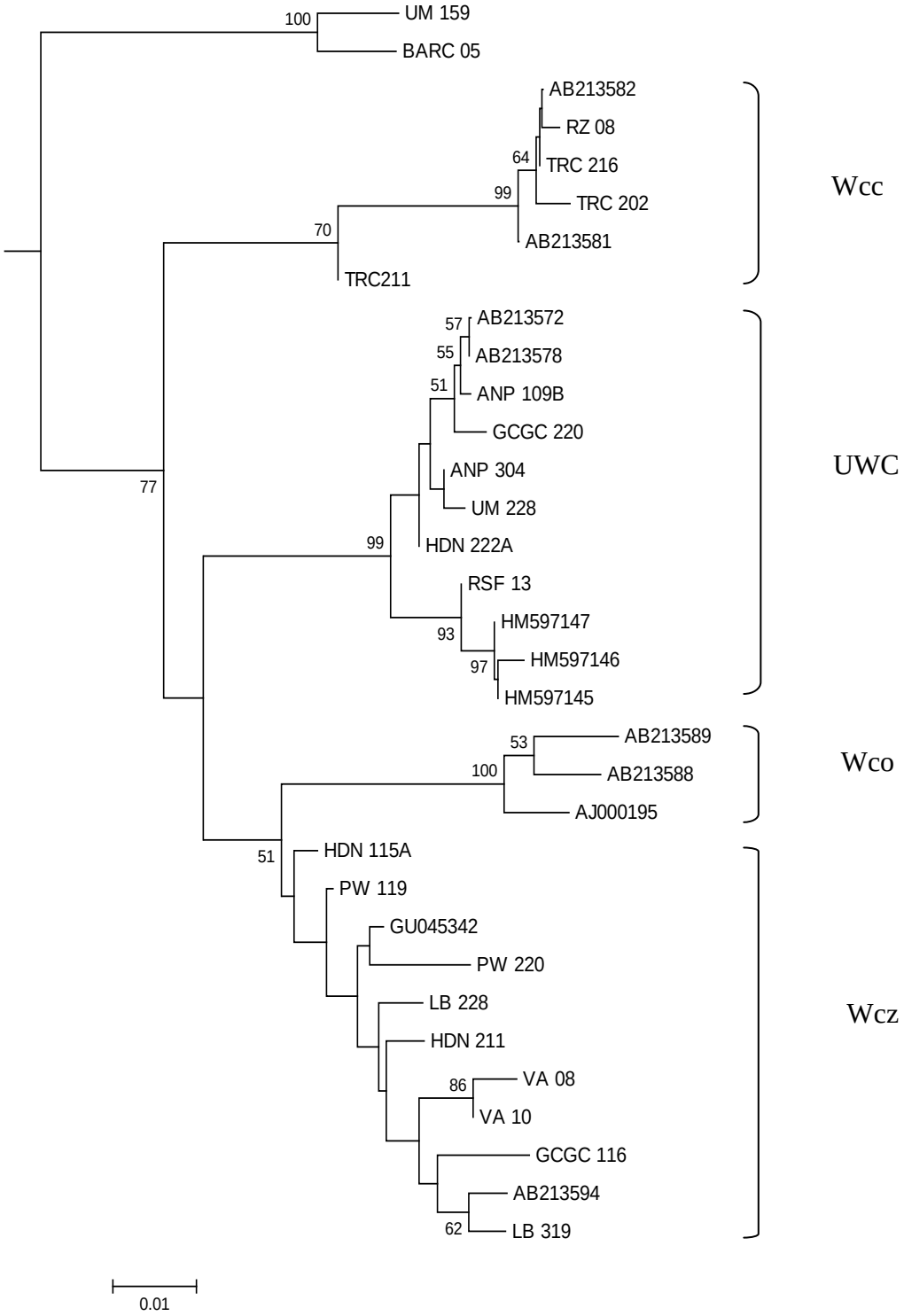


Figure 2.6 The expanded neighbor joining sub-tree of *W. circinata* clade of Figure 2.3. The sub-tree consists of four clusters including *W. circinata* var. *circinata* (Wcc), *W. circinata* var. *oryzae* (Wzo), *W. circinata* var. *zeae* (Wcz) and unidentified *W. circinata* (UWC). Bootstrap values of greater than 50% calculated by replicating data set 500 times are shown next to the branches.



In contrast to ITS analysis UP-PCR grouped BARC 05 and UM 159 within Wcz clade. These two isolates had a 100% bootstrap value. The tester strains used in this study are identified by their relevant AG in the dendrogram (Figures 2.7, 2.8, 2.9).

The primer combination of L21 and L15/AS19 generated highest number of markers (n=232) for the AG 2-2IIIB clade which included 30 isolates. There were 228 polymorphic markers for AG 2-2IIIB. However, many isolates in the group shared many markers, with some polymorphic markers occurring because of a 1-3 bp difference. The lowest number of markers was from the Wcc group with four isolates. This group produced 87 markers for both primers with 76 being polymorphic fragments. AG 1-IB group revealed 228 fragments with 2 monomorphic bands across all 18 isolates. Wcz and UWC generated 161 and 107 fragments respectively. Wcz shared two common fragments for all isolates while UWC had 4 common bands.

AFLP analysis

A total of three *Eco*RI+3 and *Mse*I+3 selective primer combinations were tested (see Table 2.2) for the ability to group *Rhizoctonia* isolates into their infraspecies level. All three primer combinations generated numerous fragments which were visualized as peaks with varying amplitudes in GeneMapper® software. The settings of the software were changed to score only distinct and clear peaks and to avoid background noise due to stutter peaks. Results of primer pair *Eco*RI-*AGT* and *Mse*I-*CTG* were not included in the analysis since this primer combination reduced the grouping ability with respect to AGs and AG subgroups of tested isolates. The primer pair *Eco*RI-*AAC* and *Mse*I-*CAC* revealed a total of 170 bands ranging from 50 to 500 bp. The *Eco*RI –*AAA* and *Mse*I-*CTA* primer combination produced 213 fragments. All fragments were polymorphic across the *Rhizoctonia* isolates.

The dendrogram clustered most of the tested isolates into their AG subgroup level (Figure 2.10). However, a few isolates did not group according to their AG due to too many unique polymorphic fragments or too few common fragments generated. Three isolates of BNR, namely ANP107, BARC17 and PW249 did not group with the rest of the BNR isolates. UP-PCR analysis also did not cluster BARC17 nor PW249 with rest of the BNR isolates. Two isolates of UWC (ANP109B and GCGC220) also grouped separately, close to an AG 5 isolate. All other *R. solani* and *Waitea* isolates clustered in accordance to their AG or subgroup classification. The bootstrap values of the clades were not as high as those in the UP-PCR dendrogram indicating

Figure 2.7 Dendrogram for isolates of *Rhizoctonia* species based on neighbor-joining using P distance parameter of MEGA 4. *R. solani* AG 2-2 and *W. circinata* clades [*W. circinata* var. *zeae* (Wcz), *W. circinata* var. *circinata* (Wcc) and unidentified *W. circinata* (UWC)] have been compressed in order to show the whole tree. Data are based on 753 binary markers generated by two UP-PCR primers (L21 and L15/AS19). Bootstrap values were calculated by resampling data set 500 times.

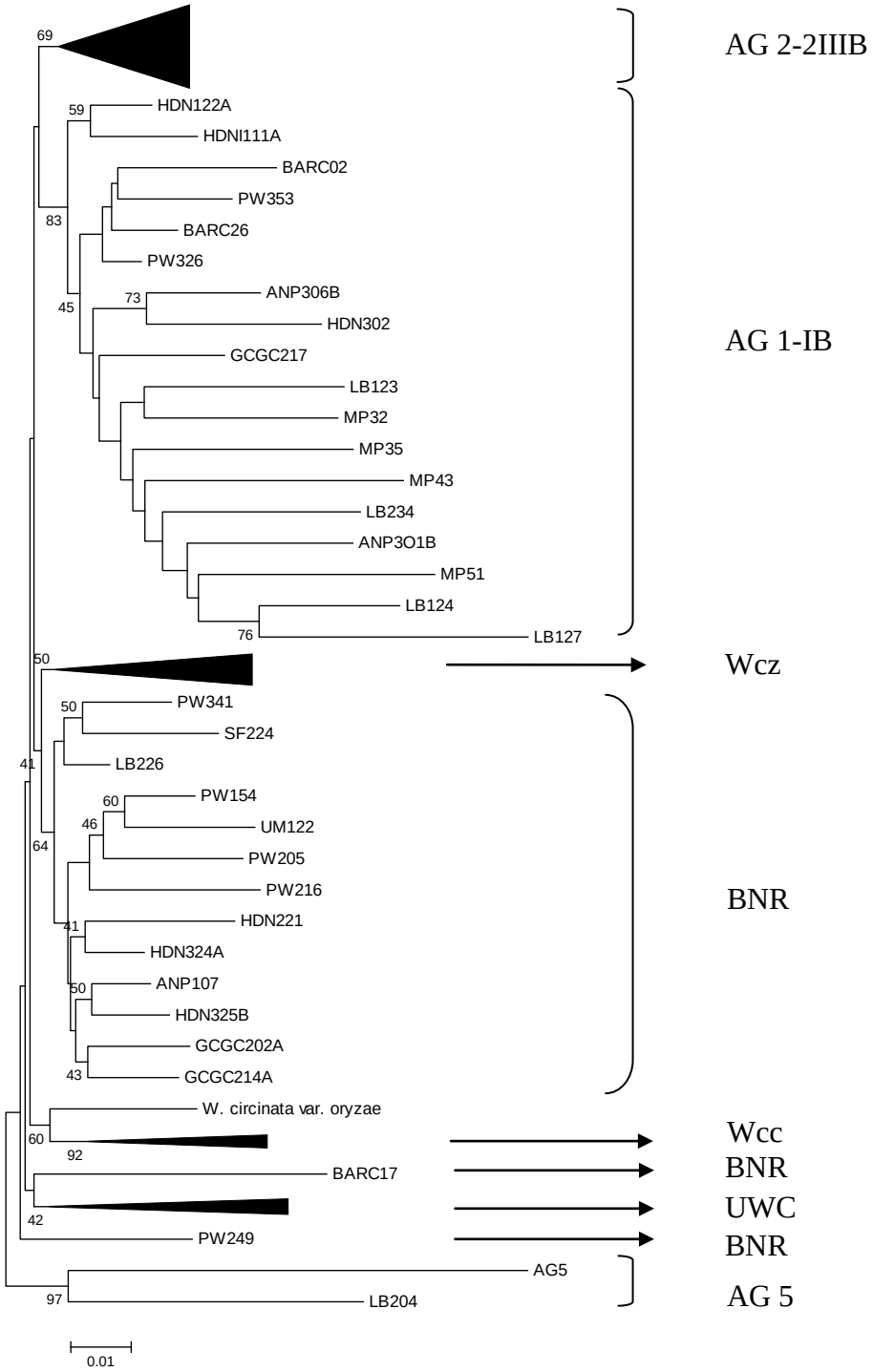


Figure 2.8 Detailed sub-tree of the compressed AG 2-2IIIB clade from Figure 2.7. The taxon AG 2-2IIIB is a tester strain.

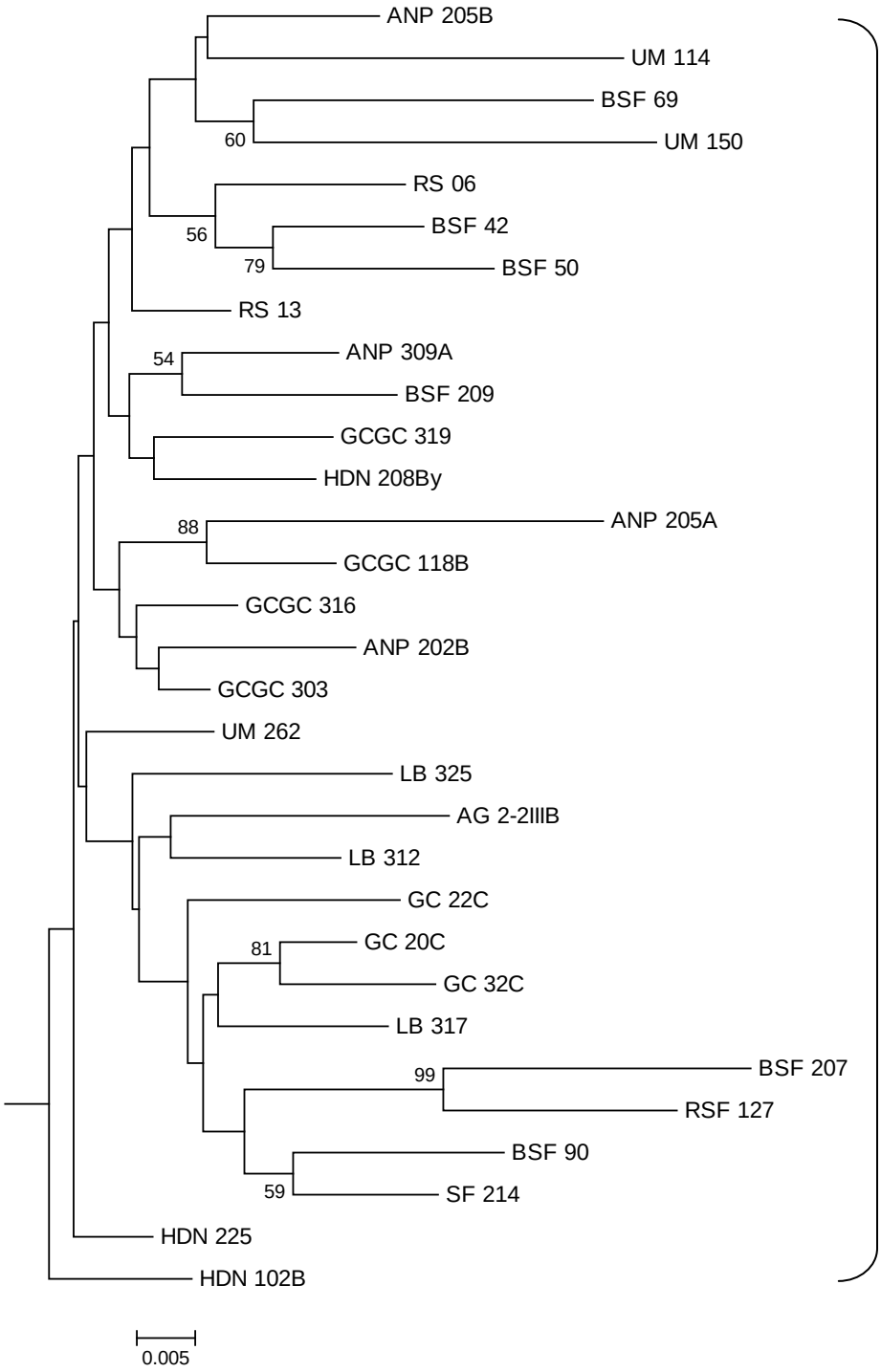
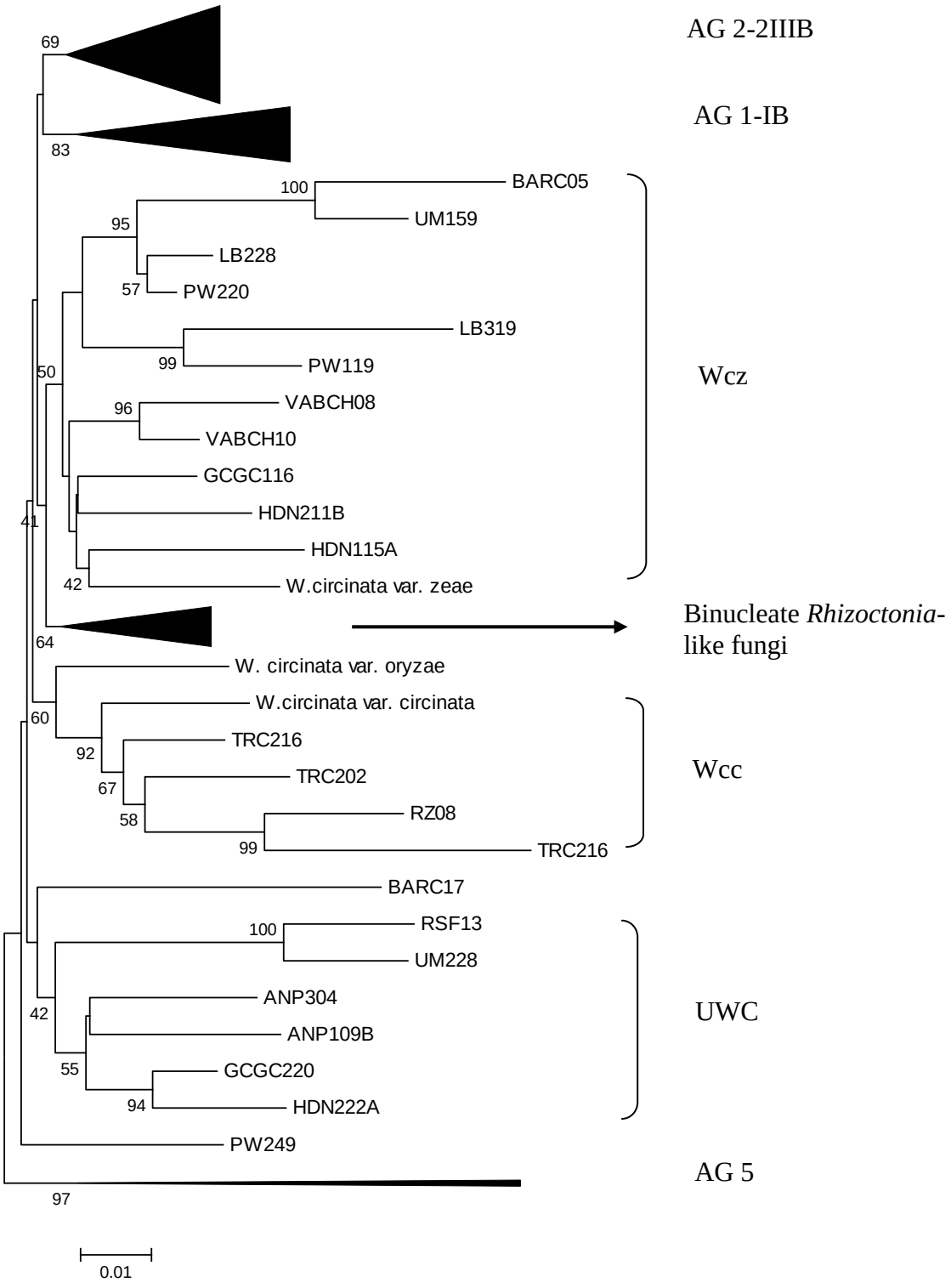


Figure 2.9 Expanded clades of *W. circinata* var. *zeae* (Wcz), *W. circinata* var. *circinata* (Wcc) and unidentified *W. circinata* (UWC) from neighbor-joining dendrogram shown in Figure 2.7. Tester strains of *Waitea* species used in the analysis are mentioned in the tree.



less reliability of the clades. Similar to UP-PCR, most isolates within an AG shared many bands though they were not monomorphic across all isolates. Within a cluster some isolates produced fragments that differed by just a few basepairs. It is notable that several fragments belonging to genetically distant groups had comigrated since group totals are higher than total fragments produced by each primer combination (Table 2.6). For instance, *R. solani* AG 1 and AG 2-2 groups had eight common fragments with primer combination *Eco*RI-*AAC* and *Mse*I-*CAC*. Primer combination *Eco*RI –*AAA* and *Mse*I-*CTA* revealed 35 common fragments for the two AGs. The BNR and Wcz groups had 33 fragments of the same length with both primer combinations.

Since AFLP primer combinations could resolve isolates of *R. solani* AG 1-IB, AG 2-2IIIB and AG5 correctly, a separate analysis was done to find out whether there was an increase in the reliability of clades when *Waitea* and BNR isolates were removed. The dendrogram (Figure 2.11) clustered isolates of different *R. solani* AGs into separate clades with comparatively high (>50%) bootstrap values.

Table 2.6 Number of AFLP fragments revealed by primer combinations *Eco*RI –*AAC/ Mse*I-*CAC* and *Eco*RI –*AAA/ Mse*I-*CTA* for main clusters of the dendrogram shown in Figure 2.9

Primer	*Eco*RI- AAC/*Mse*I-CAC*		*Eco*RI- AAA/*Mse*I-CTA*	
	Total	Common	Total	Common
AG 1-IB	51	1	90	0
AG 2-2IIIB	41	0	81	0
AG 5**	10	n/a	19	n/a
BNR	49	0	100	0
Wcc	7	1	29	2
UWC	42	0	35	0
Wcz	46	0	49	1
Total	246		403	

* When all isolates were considered together, *Eco*RI- AAC/*Mse*I-CAC and *Eco*RI- AAA/*Mse*I-CTA primer combinations revealed only 170 and 213 fragments respectively
** Represented by one isolate

Figure 2.10 Dendrogram for isolates of *Rhizoctonia* species based on amplified fragment length polymorphism (AFLP) fragment analysis. Tree construction was based on neighbor-joining algorithm using MEGA 4 software. *Rhizoctonia solani* AG 1 and *W. circinata* clades [*W. circinata* var. *zeae* (Wcz), *W. circinata* var. *circinata* (Wcc) and unidentified *W. circinata* (UWC)] have been compressed in order to show the whole tree. Data are based on 383 binary markers generated by two primer combinations *Eco*RI- AAC/*Mse*I-CAC and *Eco*RI- AAA/*Mse*I-CTA.

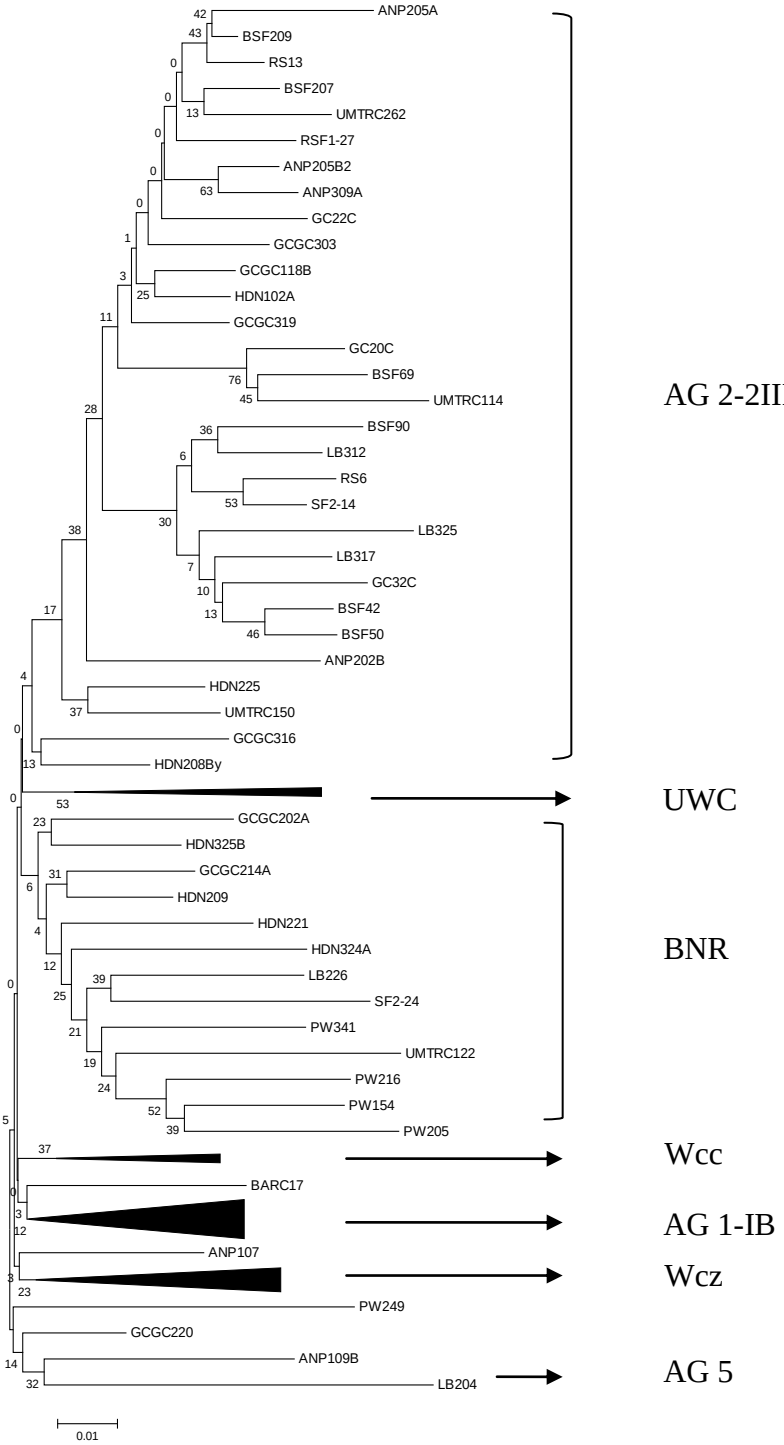
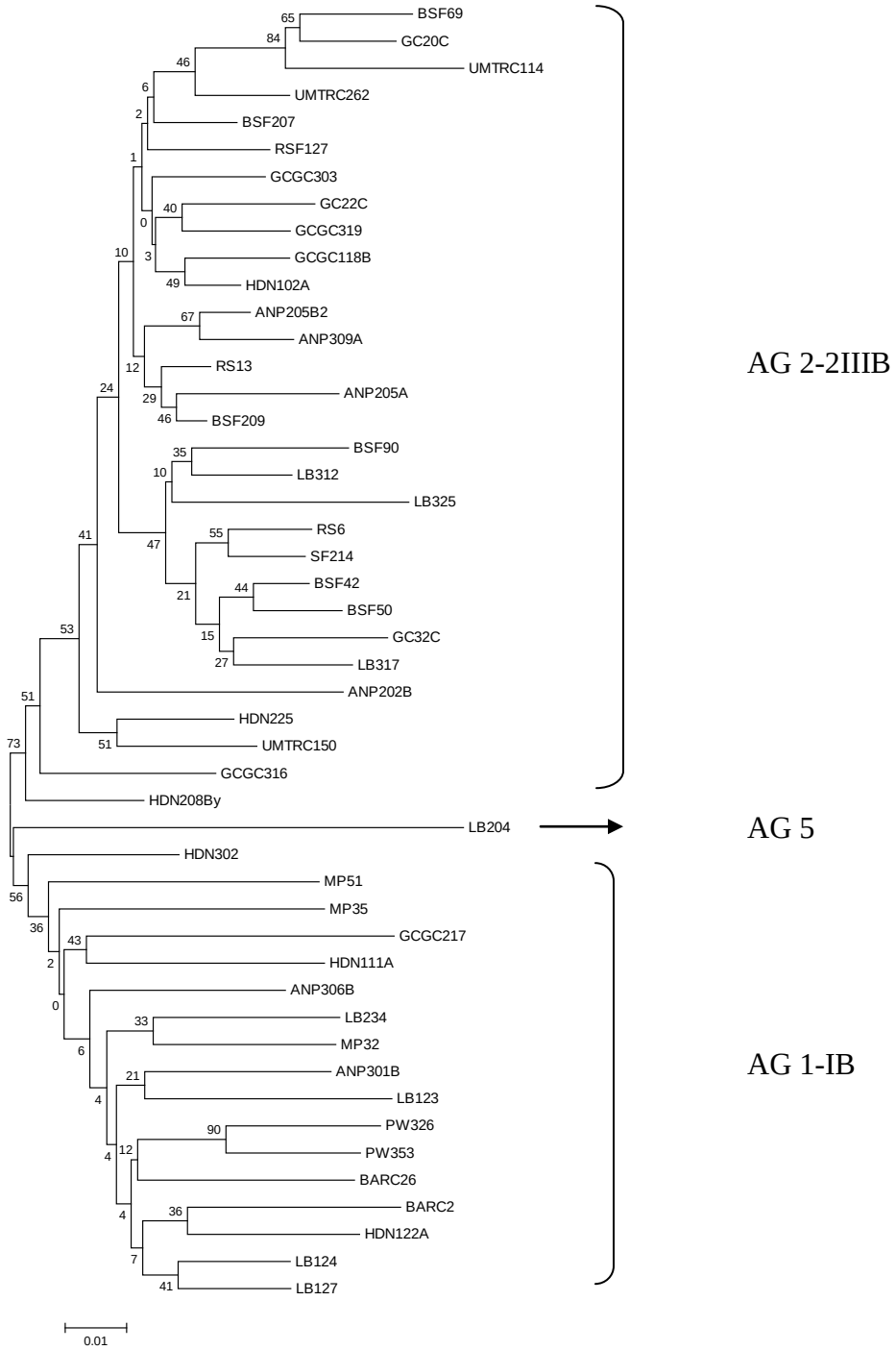


Figure 2.11 Dendrogram for isolates of *Rhizoctonia solani* AG 1, AG 2 and AG 5 based on amplified fragment length polymorphism (AFLP) fragment analysis. Tree construction was based on neighbor-joining algorithm using MEGA 4 software. Data are based on 242 binary markers generated by two primer combinations *Eco*RI- AAC/*Mse*I-CAC and *Eco*RI- AAA/*Mse*I-CTA.



Isolate composition

The isolate identification revealed *R. solani* AG 2-2IIIB to be the most common pathogen causing *Rhizoctonia* blight in the surveyed areas. Isolates of AG 2-2IIIB consisted of 35% (30/86) of the total sample. This was followed by isolates of AG 1-IB representing 21% (18/86) of the sample. Isolates of *R. solani* AG 2-2IIIB were identified from all geographic areas other than Woodbridge and Virginia Beach. Isolates of AG 1-IB were present in all surveyed locations except for Virginia Beach and Richmond (Table 2.1). *Rhizoctonia solani* AG 5 was the least common pathogen among sampled turfgrasses. A single isolate was identified from Leesburg. *Waitea circinata* var. *zeae* (Wcz) was the most dominant *Waitea* species present in the surveyed areas. Wcz had a representation of 13% (11/86). Isolates of Wcz were present in low numbers in all locations except Blacksburg and Richmond (Table 2.1). All isolates of *W. circinata* var. *circinata* (Wcc) (n=4) were identified from the Virginia Tech turfgrass research center. In Virginia, this species was first reported in 2008 from golf courses in Reston (18). However, in the present study infected turfgrasses sampled close to Reston (i.e. Herndon and Leesburg) did not reveal any isolates of Wcc. Unidentified *W. circinata* (UWC) isolates (n=6) indicate that there may be different varieties of *W. circinata* species infecting turfgrasses at very low populations. Isolates of UWC was identified from Herndon, Leesburg and Richmond of VA, and Beltsville and Annapolis of MD. Binucleate *Rhizoctonia*-like fungi (BNR) also had a presence in most locations and represented 18% (16/86) of the sample. However, isolates in the sample from Blacksburg and Virginia Beach did not reveal any BNR (Table 2.1). It is possible that they don't contribute much to disease epidemics since earlier studies reported BNR from turfgrasses and soil to be weakly pathogenic or non-pathogenic (6; 17). There were no binucleate *R. cerealis* isolates in the sample. *R. cerealis* is a cool weather pathogen and therefore it would have been unlikely for this pathogen to infect turfgrasses and be isolated during the summer when our samples were collected.

The major pathogen of all surveyed locations except for Herndon, Woodbridge and Virginia Beach was *R. solani* AG 2-2IIIB. The site surveyed in Virginia Beach appeared to dominate by *W. circinata* var. *zeae*. Binucleate *Rhizoctonia*-like fungi dominated the Herndon and Woodbridge areas. In most of the cases, it was possible to identify more than one *Rhizoctonia* species or AG from a single site. For example one site in Beltsville revealed isolates of *R. solani* AG 2-2IIIB, BNR and Wcz (UMTRC 114, 150, 122 and 159 in Table 2.1). Isolates

from another site in Leesburg were designated AG 1-IB, AG 5, BNR and Wcz (LB 234, LB 226, LB 204 and LB 228 in Table 2.1). However some sites were dominated with a single *Rhizoctonia* species. For instance all BSF isolates (Table 2.1) came from a single site in Richmond. *Rhizoctonia* isolates of all these sites were obtained from diseased tall fescue lawns.

Discussion

Zhang and Dernoeden (1992-1993) surveyed perennial ryegrass (PRG) (*Lolium perenne*), colonial bentgrass (*Agrostis tenuis*), tall fescue, creeping bentgrass, and Kentucky bluegrass for brown patch causal agents in Maryland (47). The majority of samples were collected from the University of Maryland turfgrass research facility. Over 70% (22/30) of the total isolates from PRG were AG 1-IA followed by 20% (6/30) of isolates being AG 2-2IIIB. Two isolates belonged to AG 4, and most of the *R. solani* isolates from colonial and creeping bentgrass were AG 2-2IIIB. Tall fescue and Kentucky bluegrass revealed both AG 1-IA and AG 2-2IIIB isolates. The majority of diseased leaf samples collected in the present study were from tall fescue. Other isolates were collected from golf greens having creeping bentgrass and annual bluegrass (CBG\ABG). All *R. solani* isolates from CBG\ABG were AG 2-2IIIB which agrees with Zhang and Dernoeden (47). In contrast to Zhang and Dernoeden's findings (47), our present analysis did not reveal isolates of AG 1-IA from tall fescue. All isolates belonged to AG 1 subgroup IB. However, they identified subgroups of isolates of *Rhizoctonia* using anastomosis and colony morphology but did not employ any molecular techniques. It is possible that isolates identified as AG 1-IA by Zhang and Dernoeden (47) would have been identified as AG 1-IB with our molecular techniques.

Both *R. solani* AG 1-IA and IB have been reported to cause brown patch on turfgrasses. Kuninaga in Japan identified an AG 1-IB isolate from turfgrass (21). Hsiang and Dean found an isolate of *R. solani* from *Agrostis palustris* to group with the AG 1-IB clade in their ITS sequence analysis of unknown *R. solani* isolates from turfgrss (16). A survey carried out by Tomaso-Peterson and Trevathan in Mississippi identified *R. solani* AG 1-IB from tall fescue (42). All of these studies used one or two isolates from each AG and did not involve surveying of turfgrasses infected by *R. solani* to determine the predominant AG.

Though many AGs of *R. solani* can infect turfgrasses, AG 1, AG 2-2 and AG 4 are considered to be the most common (37). Martin and Lucas conducted an extensive survey of *R. solani* from cool-season turfgrasses in North Carolina (31). They reported AG 1 to dominate cool-season grasses, but AG 2-2, AG 4 and AG 5 were also isolated. However, studies done in Massachusetts revealed AG 2-2 to predominate on cool-season turfgrasses (47). *R. zeae* has also been isolated from both cool-season and warm-season turfgrasses (7; 31). However, there has not been wide documentation of *R. zeae* as a major pathogen on cool-season grasses.

The sequence analysis of the ITS region showed that isolates within AG 1-IB had the most variation. Variability within AG 1-IB was between 90.5 to 100%, while isolates from other clades had a sequence similarity ranging from 96.5-100%. These results largely agree with the ITS sequence analysis work done by Sharon et al. (2006) (35). They analyzed all the ITS sequences of multinucleate *Rhizoctonia* (*Thanatephorus* and *Waitea*) deposited in the GenBank database. According to them only AGs 7, 11 and 1-IB had a wide percent sequence similarity range (~91-100%). All other AG subgroups and AGs had a narrower range of similarity (94-100%). The ITS region had a lot of minor variations of nucleotides across the sequences. These variations are due to insertions and deletions (15; 35). Therefore, multiple alignment of ITS sequences are generally difficult and it often creates regions with ambiguous alignments. These regions were removed from both ends of the alignment before construction of the phylogenetic tree. Analysis of ITS regions clustered isolates of *R. solani, W. circinata* and BNR into well supported clades. Isolates of *R. solani* and *W. circinata* further grouped into their anastomosis subgroups or varieties. There are many publications of ITS sequence studies on *Thanatephorus* and *Waitea* species where ITS clustering supported anastomosis groups, and confirmed that anastomosis has a solid genetic basis (14-16; 21; 41). Kuninaga et al. (1997) analyzed the ITS region of 45 *R. solani* isolates belonging to AG 1 through 9 and AG-BI (21). Sequence homology of isolates within an AG subgroup was above 96%. Isolates of different subgroups within an AG had 66-100% sequence homology, and isolates from different AGs had 55-96% sequence homology. The ITS sequence analysis grouped isolates of AG 1 and AG 4 into their subgroup level, i.e, AG 1-IA, IB and IC isolates separately within AG 1 and subgroups HG-I and HG-II within AG4. However, most of the other AGs consisted of one or two isolates, which reduced the reliability of clusters and the percent sequence homology values. Sharon et al. in

their review papers on classification and identification of *Rhizoctonia* species mention the ability of ITS region analysis for grouping isolates of *Rhizoctonia* into their AG subgroups (35; 36).

Gonzalez et al. (2001) studied phylogenetic relationships of anastomosis groups of *Ceratobasidium* (binucleate *Rhizoctonia* spp.) and *Thanatephorus* (multinucleate *Rhizoctonia* spp.) isolates by ITS sequence analysis. A phylogenetic tree from ITS sequences of 122 isolates revealed 21 groups of *Thanatephorus* and 10 groups of *Ceratobasidium* that corresponded well with previously recognized AG or AG subgroups. However, a few isolates of *Ceratobasidium* grouped closely to *Thanatephorus* isolates. These relationships were not strongly supported by bootstrap values. A limitation of their analysis was to represent several AGs of BNR with only one or two ITS sequences per AG. Since percent sequence similarity of rDNA-ITS of BNR and some multinucleate *Rhizoctonia* isolates varies widely within AGs or subgroups it is necessary to include at least several isolates per AG or subgroup in order to increase the reliability of the analysis and to confirm the correct placement of clustered isolates (35; 36).

ITS sequence analysis was used by de la Cerda et al. (2007) in order to identify Wcc causing brown ring patch disease of annual bluegrass in the United States (14). The analysis also included deposited ITS sequences of Wcz and Wco from GenBank. Their phylogenetic tree clearly separated *W. circinata* isolates into their varieties by clustering isolates of each variety separately. Previous work supports using rDNA-ITS region analyses as a reliable method for analyzing *Rhizoctonia* species belonging to *Ceratobasidium*, *Waitea* and *Thanatephorus* teleomorphs.

Although our results demonstrated clear separation of isolates of *Rhizoctonia* into their AG subgroups, there were a few isolates clustering outside their taxonomic groups. BARC17 (BNR isolate from Beltsville, MD) clustered outside the rest of the BNR isolates. Anastomosis performed with BARC17 isolates and eight other BNR isolates which grouped together, did not result in any C3 fusions with BARC17. However, all eight isolates anastomosed one another. Therefore, BARC17 may represent a different AG from the rest of the BNR isolates. The ITS sequence analysis clustered *W. circinata* isolates UM159 and BARC05 separately from the rest of the clades of *Waitea* isolates (Figure 2.6) with 100% boot strap support. However, DNA fingerprinting techniques grouped these isolates within the Wcz clade. Sclerotial morphology of these two isolates on PDA was also similar to the Wcz isolates. These two isolates showed a high sequence dissimilarity of 8.5% with other isolates of Wcz. A reason behind this may be the

presence of different rDNA-ITS regions within these isolates. It is our experience that direct sequencing of ITS regions of *Waitea* isolates is more difficult than *R. solani* isolates. Different rDNA-ITS copies can create background and hence may result in poor quality sequencing or overlapping peaks. Pannecoucque and Höfte (2009) reported detection of rDNA-ITS polymorphism in *R. solani* AG 2-1 isolates (33). They selected four isolates for which the PCR RFLP suggested sequence heterogeneity and analyzed them by cloning. At least ten clones per isolate were sequenced to determine variation of ITS region. All isolates showed polymorphism at different levels including nine different sequences for one isolate. Mutations were mainly due to substitutions, deletions and insertions. They reported that many sequences derived from clones of the same isolate did not cluster together. ITS region analysis of the present study was done by direct sequencing and therefore cloning of BARC05 and UM159 may help to reveal differences in rDNA-ITS sequences for these and other *W. circinata* isolates.

UP-PCR fragment analysis revealed its potential to group unknown *Rhizoctonia* isolates into genetically similar groups. This method amplifies intergenic areas (phylogenetically not conserved) of genomic DNA and the resulting markers can distinguish among very similar strains better than the RAPD technique (5). Although UP-PCR was introduced in the early 1990s by Bulat et al. (4), few researchers have used this method to investigate genetic diversity of *Rhizoctonia* populations. It is clear that incorporating the results of more than one primer can enhance the ability to group *Rhizoctonia* isolates. The reliability of clusters increased greatly when multiple primers such as L21 and L15/AS19 were used. However, L15/AS19 alone could not group *Rhizoctonia* isolates correctly into their AG subgroups. Primer screening is important since banding patterns of some primers can give meaningless clusters. In this study a total of four primers (L21, L45, AS15 and L15/AS19) were tested initially for their ability to produce fragments. All tested primers were capable of producing multiple fragments and primers L21 and L15/AS19 were selected for full analysis based on the total fragments generated. Previous studies on different fungi have employed several primer combinations to obtain desirable clustering of closely related strains.

The genetic diversity of *Aschochyta pisi* isolates which are responsible for leaf, stem and pod rot on pea (*Pisum* species) was studied by Lübeck et al. (28) They applied the UP-PCR technique to group isolates of *A. pisi* using eight UP primers. Two primers were not used in the analysis since they generated numerous bands of weak amplification products. The remaining six

primers proved to be suitable for identifying genetic diversity of the isolates. A cladistic tree constructed by unweighted pair group method and arithmetic mean (UPGMA) separated isolates into two distinct groups. The main differences of this study from ours is that they used only a few isolates (n=8) and band scoring was done by visual inspection after fragment separation on 1.7% agarose gels. All six primers used for analysis revealed a total of 24 markers. In contrast to visual band scoring, digital capturing of fragments by capillary gel electrophoresis can score many distinct bands per primer with high accuracy. Our analysis scored a total of 753 markers between 50-1000 bp range with L21 and L15/AS15 primers. Danielsen and Lübeck (2010) investigated genetic diversity of isolates of the highly polyphyletic species *Peronospora farinosa* causing downy mildew on seed crop quinoa (*Chenopodium quinoa*) (13). Authors were able to group isolates of the pathogen according their geographic origin by using results of three UP primers viz. L21, AS15 and L45. No clusters revealing proper geographic origin were generated when these three primers were excluded from the analysis. These studies support the necessity for screening primer combinations to obtain best results. Isolates in our analysis did not form clusters within AG subgroups that correspond with their geographic origin.

It should be emphasized that some scientists consider fragments obtained from UP-PCR and other fingerprinting techniques to be phenotypes rather than genotypes (10; 24). The reason for this is the ability of two fragments of same base pair length derived from two genetically unrelated isolates to comigrate and produce bands at the same location. As a result cladograms constructed by analyzing DNA banding patterns are not phylogenetic but phenetic in nature. The adjacent clusters of these trees do not necessarily represent evolutionarily closer species or isolates. For instance, the BNR cluster in the UP-PCR derived tree is in between Wcz and Wcc. Two BNR isolates BARC17 and PW249 (from Woodbridge, VA) grouped outside of the other BNR isolates. This result indicates a large genetic distance of these two isolates from the other binucleates. However, when only one or two isolates represent an AG group or subgroup, cladistic analysis may locate these isolates incorrectly (35; 36). This is one possible reason that our tree did not group these two isolates with other BNR isolates.

The AFLP method produced numerous bands for each individual isolate. Of these only distinct and clear bands between 50-500 bp were scored to derive genetic relatedness of the investigated isolates. One advantage of AFLP over UP-PCR or RAPD is that the number of fragments amplified from genomic DNA can be influenced in the selective amplification step.

The higher the number of selective nucleotides in the primer combination, the fewer the number of loci amplified (44). We used three selective nucleotides for each primer to prevent generating an excessive number of fragments. Too many fragments could reduce the resolving power of isolates into genetically related groups by forming numerous meaningless clusters. DNA fingerprinting techniques in general are suitable for deriving genetic differences of closely related *Rhizoctonia* individuals within an AG or AG subgroups (11; 35). The current study investigated *Rhizoctonia* isolates not only from different AGs but also from different genera (*Thanatephorus, Ceratobasidium* and *Waitea*). It is possible that AFLP may have yielded unsuitable results because we analyzed all the isolates together, but one of the aims of this study was to compare the relative efficacy of two fingerprinting techniques, AFLP and UP-PCR, in grouping unknown isolates of *Rhizoctonia*. Therefore, we decided that all isolates would be included in the analysis.

The AFLP analysis revealed a high level of heterogeneity among some isolates of BNR and UWC. Two isolates of UWC and three isolates of BNR clustered separately from the respective groups. However, all other isolates of *Rhizoctonia* grouped according to their AG subgroups or varieties. When the analysis was performed only for AGs of *R. solani* (i.e. AG 1, AG 2 and AG 5), the dendrogram constructed produced more reliable branches. This indicates the suitability of AFLP for the analysis of closely related isolates. Screening more primer combinations may yield better fragment polymorphisms across the isolates and result in a more reliable grouping based on known characters like AGs and AG subgroups.

The AFLP technique was used in a study done in India to characterize 110 *Rhizoctonia* isolates causing rice sheath blight (39). Conventional techniques and rDNA analysis revealed three different *R. solani* AG 1 subgroups (i.e. IA, IB and IC) and *R. oryzae-sativae* as the causal agents. Authors prescreened a total of 64 *Eco*RI+2/*Mse*I+2 selective primers with a sample representing the two species and AG subgroups to select the best primer combinations. Most selective primer combinations generated too many fragments for reliable scoring. The authors selected four primer pairs based on the polymorphism rate observed, the number of bands obtained, and the clarity of amplified products for the analysis of the complete set of isolates. AFLP cluster analysis grouped *R. solani* subgroups separately and differentiated *R. solani* from *R. oryzae-sativae*. Further, they also found most isolates of AG 1-IA (main causal agent, n=96) to sub-cluster according to their geographic origin. Our analysis did not show clusters associated

with geographic origin of the isolates. Lopez-Olmos et al. (2005) performed AFLP on nine isolates of *R. solani* from common bean (*Phaseolus vulgaris*) (23). They analyzed 17 tester strains representing different AGs and AG subgroups along with the unknown isolates. Four primer pairs were used for the analysis. The cluster analysis grouped unknown isolates with AG 1-IB, AG 2-3 and AG 5 tester strains. The investigated isolates (n=9) generated a total of 412 fragments, of which only three fragments were monomorphic. Our results are similar as there were no monomorphic bands across all isolates and only a few common fragments were observed among isolates within the same AG. The study done by Lopez-Olmos et al. (2005) demonstrated a close relationship between AFLP genotype and AG (23).

AFLP is a powerful tool to derive subtle, cryptic relationships of closely related isolates but may not be effective at analyzing isolates of different species or AGs together. A study done by Ceresini et al. (2002) demonstrated AFLP could cluster *R. solani* AG 3 isolates from potato (AG 3-PT) and tobacco (AG 3-TB) fields into two groups (10). According to authors, isolates of AG 3 from potato and tobacco plants are somatically related based on anastomosis reactions and taxonomically related based on fatty acid, isozyme analyses and DNA characters. However, according to them, there is also evidence of considerable differences in their biology, ecology and epidemiology. Ceresini et al. performed AFLP with two selective primers without any prior primer screening (10). The dendrogram constructed by AFLP fragment analysis separated isolates of AG 3-PT and AG 3-TB into two clades. AFLP analysis also revealed all tested AG 3-PT isolates (n=32) had different AFLP fingerprinting patterns (phenotypes) whereas several clones (same fingerprinting pattern) were found among 36 AG 3-TB isolates investigated. Therefore it is possible the AFLP technique may be more useful in deriving relationships of isolates of *Rhizoctonia* within an AG rather than between *Rhizoctonia* species or multiple AGs.

Different molecular techniques viz. rDNA-ITS sequences, UP-PCR and AFLP clustered investigated isolates largely according to their AG or AG subgroup. We did not see any correlation of sampling site influencing clusters formed. However, it seems apparent that different turfgrass management programs had influenced the genetic structure of isolates of *Rhizoctonia* to a certain extent. All *R. solani* isolates from putting greens with creeping bentgrass and annual bluegrass (CBG\ABG) were designated AG 2-2IIIB while all AG 1-IB isolates were from tall fescue lawns or golf course roughs (Tables 2.1 and 2.3). However, AG 2-2IIIB was also isolated from tall fescue. Unlike lawns or roughs, putting greens are intensively managed with

routine fungicide spray programs. However we do not have specific fungicide management history, and other cultural management programs such as mowing, fertilization and irrigation for each location surveyed. Further studies are needed to ascertain whether AG 2-2IIIB is better adapted to putting green conditions or prefers CBG/ABG turfgrasses compared to AG 1-IB isolates.

Our analysis showed that different *Rhizoctonia* species and AGs have the potential to infect cool-season turfgrasses. It is apparent that predominant causal agent of extensively managed CBG\ABG putting greens is the *R. solani* AG 2-2IIIB. Our sample contained only 16% (14/86) of isolates from putting greens. Therefore, it is useful to characterize more *Rhizoctonia* pathogens from putting greens to test the dominance of AG 2-2IIIB isolates. The major pathogen from the majority of the locations surveyed was AG 2-2IIIB followed by AG 1-IB. Future research may use molecular techniques performed in this study to understand how *Rhizoctonia* populations change over different seasons at a single location. It is also interesting to find activity of pathogens adapted to different climatic conditions such as *R. cerealis* (cool weather pathogen) and *R solani* (warm weather pathogen) by surveying the same site at short intervals from mid spring to early autumn.

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CHAPTER 3

Identification of *Rhizoctonia* strains by UP-PCR cross blot hybridization and primers based on unique UP-PCR markers

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ABSTRACT

A rapid identification assay for *Rhizoctonia* isolates causing patch diseases on turfgrasses was developed based on universally primed polymerase chain reaction (UP-PCR) product cross blot hybridization method. Tester strains belonging to different *Rhizoctonia* anastomosis groups (AGs) and subgroups were amplified with a single UP primer which generated multiple DNA fragments for each isolate. Probes were prepared with fragments specific to each tester strain by labeling with digoxigenin. Target *Rhizoctonia* isolates were amplified with the same UP primer and spotted onto a filter, immobilized and used for cross-hybridization against amplification products of labeled tester strains. Isolates within an AG subgroup cross hybridized strongly while isolates of different AGs did not cross-hybridize or did so weakly. *R. solani* and *W. circinata* isolates from diseased turfgrass leaves were identified using this assay. Sequence-characterized amplified region (SCAR) markers were generated from UP-PCR fragments to identify isolates of *R. solani* major pathogenic groups AG 1-IB and AG 2-2IIIB. A UP-PCR fragment unique for isolates of each group of AG 1-IB and AG 2-2IIIB was selected for designing specific primers. Depending on the specificity of the primers, a single PCR product was amplified from pure DNA for isolates of either AG 1-IB or AG 2-2IIIB groups. Pure DNA of none target isolates of *Rhizoctonia* did not give any PCR product. Further studies are needed to test the ability of these specific primers to detect target pathogens from diseased leaves.

Introduction

Diseases caused by different *Rhizoctonia* species are generally known as *Rhizoctonia* blight. Several cool-season turfgrasses of the transition zone are susceptible to *Rhizoctonia* species. The main hosts of *Rhizoctonia* blight are tall fescue (*Festuca arundinacea* Schreb.), creeping bentgrass (*Agrostis stolonifera* L.) and perennial ryegrass (*Lolium perenne* L.) (4; 17; 26). *Rhizoctonia* blight commonly occurs during summer months and disease symptoms include necrotic irregular or circular patches of 5-60 cm (2 inches to 2 ft) on golf greens and yellow circular patches of 15-30 cm on lawns (22). Several species are responsible for causing *Rhizoctonia* blight on turf and depending upon the causal agent different common names are used to identify the disease. Brown patch caused by *R. solani* Kühn (teleomorph: *Thanatephorus cucumeris* (Frank) Donk) is the most common disease (22). *Waitea circinata* var. *zeae* and var. *oryzae* (anamorphs: *Rhizoctonia zeae and R. oryzae respectively*) are responsible for leaf and sheath spot disease on turfgrass while *W. circinata* var. *circinata* causes brown ring patch disease (22). However, disease symptoms on turfgrasses are more or less similar and visual diagnosis is not a reliable way to determine the causal pathogen. The main causal agent, *R. solani* is a species complex composed of many genetically distinct groups (6). So far 14 anastomosis groups (AGs) of *R. solani* have been established (13) and AG 1, AG 2-2IIIB, and AG 4 have been identified to occur commonly on cool-season grasses (4; 22). The traditional methods of identifying *Rhizoctonia* isolates are by cultural morphology and anastomosis reactions. However, these methods are laborious and time consuming and sometimes difficult to interpret. This makes it difficult to determine correct disease management practices. Identification of causal agents at disease initiation is very important since *Rhizoctonia* blight can spread to a large area within a short period of time provided weather conditions are favorable. The population study carried out by the author (see Chapter 2) revealed AG 2-2IIIB and AG 1-IB isolates tend to dominate brown patch infected cool-season grasses from different geographic areas of Virginia and Maryland. A significant number of *Waitea circinata* isolates were also identified in the analysis.

In recent years various molecular techniques have been employed to characterize and identify *Rhizoctonia* isolates. One such popular method is to analyze the rDNA-ITS (ribosomal DNA- internal transcribed spacer) region (13; 21). A large number of ITS sequences of *Rhizoctonia* species and AGs can be found in publicly accessible databases like NCBI (National Center for Biotechnology Information: www.ncbi.nlm.nih.gov). Once the ITS region of an

unknown *Rhizoctonia* isolate is sequenced, it can be compared with already deposited sequences for identification. This is known as a BLAST (The Basic Local Alignment Search Tool) search. Another advantage of publicly available databases is that ITS sequences of reference strains can be downloaded and incorporated with unknown sequences to construct phylogenetic trees. However, when there are many samples, DNA sequencing can be a costly exercise for most laboratories since genetic analyzers are not widely available and they can be expensive to maintain. When DNA sequencing is outsourced, it may also delay the identification process. Another disadvantage of using public databases is the potential for erroneously identified sequences or sequences having limited detail. Much of these data are unpublished and not subjected to peer review. For instance, some *Rhizoctonia* ITS sequences from the NCBI database are identified only to species or anastomosis group level and nothing is mentioned about their subgroup. Under these circumstances BLAST results may provide limited information as an identification tool. Sometimes search results may give two or more *Rhizoctonia* AGs or subgroups as having a similar ITS sequence similarity percentage to the unknown strain. In this case correct identification is problematic. Therefore developing alternative methods to identify *Rhizoctonia* species rapidly and accurately would be useful.

The present study is an attempt to develop rapid identification methods for *Rhizoctonia* isolates based on UP-PCR (Universally primed-PCR) product cross blot hybridization and primer design for unique molecular markers to identify *R. solani* AG 1-IB and AG 2-2IIIB. UP-PCR is a PCR based DNA fingerprinting technique similar to the well known RAPD (random amplified polymorphic DNA) method. This technique has the ability to generate numerous banding patterns from primarily intergenic, less conserved regions of the genome (1). UP-PCR primers are longer (15-21 nt) than RAPD primers (typically 10 nt) and therefore can anneal under more stringent conditions (52-60°C) ensuring reproducibility of banding profiles (15; 24). UP-PCR and other molecular techniques such as DNA-DNA hybridization, RAPD, analysis of ribosomal DNA (rDNA) and rDNA restriction fragment length polymorphism (rDNA-RFLP) have been used to identify and group *Rhizoctonia* strains (9; 12; 14; 19; 21). DNA fragments generated by these methods have shown low sequence similarity among different anastomosis groups (AGs) and AG subgroups. Cross blot hybridization of UP-PCR products generated with a single primer is a potential tool for the determination of sequence homology to many *Rhizoctonia* strains simultaneously (15). Cross blot hybridization is done by blotting UP-PCR products from

different strains onto a membrane and attaching labeled products of a reference strain in each blot. Probe DNA can be labeled by radioactive phosphorous or a non-radioactive molecule such as digoxigenin (DIG). The strength of the hybridization signal is used to determine the relatedness of the reference strain to the unknown one. Non-radioactive detection methods are based on either colorimetric or chemiluminescent reactions. In particular, chemiluminescence detection methods can be very sensitive and may need only a few minutes of exposure to an autoradiography film to visualize the signal of homologous products. UP-PCR cross-hybridization is much faster than the traditional method of DNA-DNA hybridization technique. The latter involves time consuming pair-wise hybridization experiments. Though DNA banding patterns are considered to behave as phenotypes, UP-PCR product cross-hybridization allows one to derive sequence homology, and therefore has a phylogenetic dimension. Analysis of UP-PCR banding profiles can also group and identify genetically related isolates. However, efficient band scoring requires loading samples into a genetic analyzer which is costly and expensive to maintain. Cross blot hybridization eliminates this hurdle. Once probes are labeled with DIG they can be used for one year without reducing the efficacy (www.roche-applied-science.com). Lübeck and Poulsen (2001) used UP-PCR cross-hybridization assay for rapid identification of *Rhizoctonia* isolates into their AGs. UP-PCR products of 21 tester isolates belonging to 11 AGs of *R. solani* were cross-hybridized against amplification products from different isolates. They found isolates within AG subgroups cross-hybridized strongly whereas different AG subgroups and AGs showed little or no cross-hybridization (14). Furthermore, sixteen *Rhizoctonia* isolates from diseased sugar beets and potatoes were identified using the assay.

The primers utilized in DNA fingerprinting methods are capable of amplifying DNA fragments from any organism and therefore cannot be used to detect a specific species or a strain from soil or infected plant parts (15). However, by identifying unique molecular markers present in the target organisms, the fingerprinting methods including UP-PCR can be used for rapid identification of organisms of interest. The essential feature of a marker is to detect all members of the target group and discriminate all others. When such markers are sequenced they are called sequence-characterized amplified regions (SCARs) (15). The sequence information of the SCAR markers can be used to synthesize primers that selectively amplify the marker and thereby act as a target site in a diagnostic assay. Previous studies have employed different molecular techniques for rapid identification of *R. solani*. Salazar et al. (2000) designed specific primers to amplify

AG 2 subgroups from rDNA-ITS sequences. However, the specific primer combination for AG 2-2 was not capable of differentiating AG 2-2IIIB,-2IV or 2LP from each other (20). A molecular marker from RAPD primer A09 was used by Toda et al. to identify AG 2-2LP from -2IIIB, -2IV and other *R. solani* AGs (23). The primer pair A091-F/R gave a single product not only from pure DNA of 2-2LP isolates but also from DNA of diseased leaf sheaths. Though there have not been any published UP-PCR SCAR markers for *R. solani*, this method has been used successfully to identify markers in other fungal organisms (2; 7).

The first objective of this study was to evaluate the efficacy of UP-PCR product cross blot hybridization to group isolates of *Rhizoctonia* from different AGs or AG subgroups. The second objective was to design specific primers for detection of *R. solani* AG 1-IB and AG 2-2IIIB from sequence data of UP-PCR products and to evaluate the uniqueness of the primers by performing PCR with pure DNA obtained from different AGs infecting turfgrasses.

Materials and Methods

Fungal isolates and DNA extraction

Twelve tester strains of *Rhizoctonia* species (Table 3.1) and isolates collected from turfgrasses (Table 2.1) were used for the cross blot hybridization study. Isolates were cultured on liquid potato dextrose broth (Difco Laboratories, Detroit, MI, USA) for 3-4 days at room temperature and DNA extracted using the protocol mentioned in Chapter 2.

UP-PCR and cross-hybridization

UP-PCR was performed twice for each tester isolate with the L21 primer and banding patterns of the UP-PCR products were visualized after electrophoresis on a 1.7% agarose gel as described in Chapter 2. Isolates which gave partially amplified DNA products or inconsistent banding patterns were discarded and UP-PCR repeated to obtain a good and consistent set of PCR fingerprints. Probe preparation and cross blot hybridization was carried out using the DIG High Prime DNA Labeling and Detection Kit (Roche Applied Sciences, Mannheim, Germany).

Table 3.1 List of *Rhizoctonia* tester strains

Isolate	Species[a]	AG	Host	Origin	Obtained from[b]
92`AR Soy	Rs	AG 1-IA	Soybean	AK, USA	CR
CR1	Rs	AG 1-IB	unknown	AK, USA	CR
BM2	Rs	AG 1-IB	Unknown	Unknown	BM
BM3	Rs	AG 1-IC	Unknown	Unknown	BM
Rh146	Rs	AG 2-2IIIB	Bentgrass	GA, USA	LB
Rh141	Rs	AG 2-2LP	Zoysiagrass	IL, USA	LB
1AP	Rs	AG 3	Unknown	Unknown	DL
RS23	Rs	AG 4	Unknown	Unknown	DL
Rh102	Rs	AG 5	Unknown	Unknown	LB
IOEEA	Rs	AG 6	Sweet gum	NC, USA	MC
EDHGED	Wcc	not assigned	ABG[c]	CA, USA	FW
AVGCAV	Wcz	WAG-Z	ABG	CA, USA	FW
87NE9	Wco	WAG-O	Rice	Japan	MC
C610	Rc	AG-D	Unknown	Unknown	DL

[a]Rs: *R. solani*, Wcc: *W. circinata* var. *circinata*, Wcz: *W. circinata* var. *zeae*, Wco: *W. circinata* var. *oryzae*, Rc: *R. cerealis*
[b]CR: Craig Rothrock, USDA-ARS, AR, BM: Bruce Martin, Clemson University, SC, LB: Lee Burpee, University of Georgia, GA, DL: Dilip Lakshman, USDA, Beltsville, FW: Frank Wong, University of Riverdale, University of California Riverside, CA, MC: Marc Cubeta, North Carolina State University, NC
[c]ABG: Annual bluegrass

Probes were prepared from tester strains of AG 1-IB, 2-2IIIB, AG 5, Wcc and Wcz (Table 3.1). UP-PCR products were purified with QuickStep™2 PCR Purification Kit (Edge Bio Systems, Gaithersburg, MD) and 30-50 ng were dissolved in 16 µl sterile distilled H_2O. PCR products were denatured by heating for 10 min at 95°C and quickly chilled on ice. Four microliters of DIG-High Prime (Roche Applied Sciences, Mannheim, Germany) was added to each tube, mixed well, and incubated overnight in a 37°C water bath to label the probe DNA with digoxigenin (DIG). The reaction was stopped by adding 2 µl of 0.2 M EDTA to each tube and DNA concentrations were quantified (usually 700-1100 ng/µl). Efficiency of DIG-labeled probes was determined by applying a series of dilutions (1 ng to 1 pg) on a nylon membrane and detecting the strength of the signal. Dilutions of DIG labeled probes were carried out by adding DNA dilution buffer containing 100 µg/ml fish sperm. This test also included manufacturer provided DIG-labeled positive control and a negative control containing only DNA dilution buffer. The nylon membrane was subjected to immunological detection with an anti-digoxigenin-

AP (alkaline phosphatase) conjugate and CDP-Star substrate. CDP-Star is an extremely sensitive chemiluminescent substrate for AP. Light emission was recorded on X-ray films. The probes which gave a significant signal for at least 100 pg blots were used for the experiment. Others were discarded and fresh probes were synthesized.

The target DNA templates (UP-PCR products of *R. solani* and *W. circinata*) were diluted with DNA dilution buffer to obtain 25 ng/µl solutions. Diluted DNA solutions were denatured by heating for 10 min at 95°C and rapidly cooled on ice. One microliter of DNA products from AG 1, AG 2, AG 5 and *W. circinata* isolates (Table 2.1) were spotted separately on positively (+) charged Amersham Hybond™-N+ nylon membranes (GE Healthcare UK Ltd, Buckinghamshire, England). Tester strains of different AGs (Table 3.1) were also blotted on each membrane alongside of target DNA products. Once the spots dried completely, membranes were placed on Whatman 3 MM blotting papers soaked with 10x SSC (saline sodium citrate) and crosslinked with a Stratalinker® UV crosslinker (Stratagene, La Jolla, CA, USA). Approximately 100,000 µJ/cm^2 energy was applied to fix the DNA to the membrane. After crosslinking, membranes were washed in sterile distilled water and dried on a filter paper. A prehybridization step was carried out by inserting each membrane in a sealed plastic bag with DIG Easy Hyp solution (Roche Applied Sciences) and shaking for 30 min in a 39°C water bath. For each 100 cm^2 membrane, 20 ml of preheated prehybridization solution was used. Hybridization was done by introducing denatured DIG labeled probe to the membranes and incubating for four hours with gentle agitation in a 39°C shaking water bath. The hybridization solution was prepared by mixing probe with 5 ml of DIG Easy Hyb to obtain a final probe concentration of 30 ng/ml. The first stringency wash was done by transferring the membranes to a container with a 30 ml solution of 2x SSC/0.1% SDS (sodium dodecyl sulfate) and then shaking it for 5 min twice at room temperature. The second wash was carried out for 20 min in a solution of 0.5x SSC/0.1% SDS for two times. This was performed in a shaking water bath at 70°C. All subsequent incubations for immunological detection steps were carried out on a shaker at room temperature (~25°C) using the reagents supplied by Roche Applied Sciences. Membranes were rinsed in 20 ml of washing buffer (0.1 M maleic acid, 0.15 M NaCl, and 0.3% (v/v) Tween 20) for 5 min and incubated for 30 min in 100 ml of blocking solution (purified fraction of milk powder in maleic acid buffer). The immunological reaction was carried out by incubating membranes for 30 min with 50 mU/ml of anti-DIG-AP fragment in 30 ml of blocking buffer. After incubation,

membranes were washed twice for 15 min in 100 ml of washing buffer and equilibrated for 5 min in detection buffer (1M Tris-HCl, pH 9.5, 1 M NaCl). CDP-Star ready-to-use substrate was used for the detection of the hybridization reactions. The membranes were placed on a transparent plastic sheet and 1 ml of CDP-Star was added and immediately covered with another plastic sheet and the substrate was spread evenly. After incubating for 5 min excess liquid was squeezed out and the edges were sealed. Membranes were exposed to X-ray films for 2-10 min and then the films were developed.

Identification of UP-PCR SCAR markers

Isolates used for screening UP-PCR primers for SCAR markers are listed in Table 3.2. Genomic DNA of the isolates was extracted as described in Chapter 2. Eleven isolates of AG 2-2IIIB and 10 isolates of AG 1-IB were tested with closely related AG subgroups to detect unique molecular markers. For this purpose UP-PCR products of AG 2-2IIIB were compared with DNA products of AG 2-2LP isolates and AG 1-IB products were compared with both AG 1-IA and -IC. UP-PCR was performed as described in Chapter 2. PCR products of each isolate were resolved in 1.7% agarose gels and visualized under UV light after staining with ethidium bromide. For designing specific primers, a unique band for AG 2-2IIIB and AG 1-IB were identified by visually inspecting different banding profiles generated by four primers (Table 3.3). For AG 1-IB, a single PCR fragment amplified by UP primer L21 was selected. For AG 2-2IIIB, a distinct PCR product was selected from UP primer L45. The gel bands of selected products were excised using a scalpel and DNA extracted using a QIAquick Gel Extraction Kit (Qiagen Inc, Valencia, CA). Invitrogen Topo TA Cloning Kit was used to clone DNA products with electrocompetent *Escherichia coli* cells (Invitrogen Corporation, Carlsbad, CA, USA). Eight transformed bacterial colonies were selected per fungal DNA fragment and tested for inserts by PCR. One confirmed transformant per fragment was cultured in 50 ml of LB liquid medium (Luria-Bertani broth) and plasmids were purified using a Qiagen Plasmid Midi Kit (Qiagen Inc, Valencia, CA, USA). Sequencing of the inserts was done using a BigDye 3.1 sequencing kit (Applied Biosystems, Foster City, CA, USA) and analyzed with ABI 3730 DNA sequencer (Applied Biosystems).

Table 3.2 List of *R. solani* isolates used in identifying SCAR markers

Isolate number	Isolate name	AG	Host	Origin
IIIB-1	UMTRC 150	AG 2-2IIIB	Tall fescue	Maryland, USA
IIIB-2	RS 13	AG 2-2IIIB	CBG/ABG	Virginia, USA
IIIB-3	GC 32C	AG 2-2IIIB	CBG/ABG	Virginia, USA
IIIB-4	BSF 42	AG 2-2IIIB	Tall fescue	Virginia, USA
IIIB-5	SF 214	AG 2-2IIIB	Tall fescue	Virginia, USA
IIIB-6	RSF 127	AG 2-2IIIB	Tall fescue	Virginia, USA
IIIB-7	LB 312	AG 2-2IIIB	Tall fescue	Virginia, USA
IIIB-8	GCGC 316	AG 2-2IIIB	CBG	Virginia, USA
IIIB-9	ANP 205A	AG 2-2IIIB	Tall fescue	Maryland, USA
IIIB-10	HDN 208By	AG 2-2IIIB	Tall fescue	Virginia, USA
IIIB-11	Rh146	AG 2-2IIIB	Bentgrass	Georgia, USA
LP-12	Rh141	AG 2-2LP	Zoysiagrass	Illinois, USA
LP-13	ACC3[a]	AG 2-2LP	Zoysiagrass	Kansas, USA
LP-14	CGCF18[2][a]	AG 2-2LP	Zoysiagrass	Kansas, USA
LP-15	SGGC2[a]*	AG 2-2LP	Zoysiagrass	Kansas, USA
IB-16	BARC 26	AG 1-IB	Tall fescue	Maryland, USA
IB-17	MP 51	AG 1-IB	Tall fescue	Virginia, USA
IB-18	LB 124	AG 1-IB	Tall fescue	Virginia, USA
IB-19	LB 234	AG 1-IB	Tall fescue	Virginia, USA
IB-20	PW 353	AG 1-IB	Tall fescue	Virginia, USA
IB-21	HDN 122A	AG 1-IB	Tall fescue	Maryland, USA
IB-22	GCGC 217A	AG 1-IB	Tall fescue	Maryland, USA
IB-23	ANP 306B	AG 1-IB	Tall fescue	Maryland, USA
IB-24	CR1	AG 1-IB	Unknown	Arkansas, USA
IB-25	BM2	AG 1-IB	Unknown	Unknown
IA-26	92`AR Soy2	AG 1-IA	Soybean	Arkansas, USA
IA-27	92`AR Soy3	AG 1-IA	Soybean	Arkansas, USA
IC-28	BM3	AG 1-IC	Unknown	Unknown
IC-29	CR2	AG 1-IC	Unknown	Unknown

[a]Provided by Megan Kennelly, Kansas State University, KN
*Represented only for specific primer screening

The specific primer pairs (Table 3.4) for AG subgroups 1-IB and 2-2IIIB were designed from the sequence data obtained with the ABI 3730. All primers were synthesized by Eurofins MWG Operon, Huntsville, AL, USA. These primers were tested with relevant *R. solani* AG subgroups (i.e. AG 1-IA,-IB and -IC and AG 2-2IIIB and -2LP) and other *R. solani* AGs, *R. cerealis* and *Waitea circinata* varieties. Amplification reactions using specific primer pairs L21-F/G and L45-A/C were performed using 50 ng of pure DNA. The other components of the PCR

reaction mixture were similar to the reaction mixture used for the UP-PCR technique. To determine the optimum PCR conditions different annealing temperatures from 58-63°C were used. The thermocycler was programmed for 2 min at 94°C followed by 35 cycles of 1 min at an annealing temperature, 30 s at 72°C, 30 s at 94°C, and one cycle of 10 min at 72°C. DNA amplifications were resolved by electrophoresis on 1.7% agarose gels. Agarose gels were stained with ethidium bromide and visualized for DNA fragments under UV light.

Table 3.3 UP-PCR primers used for investigating SCAR markers

Primer name	Sequence
L21	5´ -GGATCCGAGGGTGGCGGTTCT- 3´
L15/AS19	5´- GAGGGTGGCGGCTAG- 3´
L45	5´ -GTAAAACGACGGCCAGT- 3´
AS15inv	5´ -CATTGCTGGCGAATCGG- 3´

Table 3.4 Specific primer sequences for AG 1-IB and AG 2-2IIIB

Primer name	Sequence
L21-F	5´-TCATTGCCATCAACTGTGACT- 3´
L21-G	5´-CAAGGTTTGTTTGGAGATTGAG- 3´
L45-A	5´-ACCAATGAGTCGGTCTCGTC- 3´
L45-C	5´-CGTCTACGAGAAAACTTGCAT- 3´

Results

Cross blot hybridization

The UP primer L21 generated multiple bands of varying lengths when amplified with *Rhizoctonia* DNA. Visual examination revealed distinct banding patterns for most AGs. AG 2-2IIIB and -2LP isolates shared some bands. Figure 3.1 shows the banding profiles for several isolates of AG 1-IB and AG 2-2IIIB. Cross blot hybridization was carried out with UP-PCR products of tester strains to determine how efficiently unknown *Rhizoctonia* isolates can be identified. The isolates of *Rhizoctonia* AG 1, AG 2, AG 5 and *W. circinata* varieties listed in Table 2.1 (Chapter 2) were cross-hybridized separately with relevant tester strains to determine their hybridization signal. In each cross blot scheme, UP-PCR products of other AGs were also spotted to see whether a particular probe can distinguish them. The conditions of stringency

washes and exposure time to X-Ray film mentioned in the materials and methods were decided by trial and error to produce a strong signal for isolates homologous to the labeled probe and to reveal a minimum signal for non-homologous (different AGs) and partially (AG subgroups) homologous isolates. In this experiment my objective was to develop a cross-hybridization technique as an identification tool for *Rhizoctonia* species. Therefore, an attempt was made to avoid partial signals since these signals can make the interpretation difficult. The extreme sensitivity of the chemiluminescent substrate CDP-Star helped to increase the stringency of washes to strip probe from partially homologous target products without affecting the strength of the signal for homologous isolates.

Figure 3.2 shows the hybridization signal for AG 1-IB isolates. The labeled probe was made from UP-PCR products of strain BM2. All isolates of AG 1-IB gave significant to strong signals while other AGs and AG subgroups gave weak signals. Genomic DNA of the probe and homologous isolate MP32 were also included in the test to see whether the probe is capable of emitting a hybridization signal for genomic DNA. However, the signal was very weak (last two blots of Figure 3.2). Figure 3.3 shows the signals obtained for DNA blots of different subgroups of AG 1 when hybridized with probe BM2 (AG 1-IB). The radiograph clearly shows that strong signals are given only for AG 1-IB isolates and very weak signals for other groups. The 21 *W. circinata* isolates comprising Wcz, Wcc and unidentified *W. circinata* (UWC) (Table 2.1) were hybridized with a probe prepared from tester strain Wcz AVGCAV. All isolates of Wcz except for LB319 gave clear signals (Figure 3.4) to distinguish them from isolates of Wcc and UWC. LB319 gave a weak signal. All UP-PCR blots of other AGs did not result in a signal. When fresh UP-PCR products of LB319 were cross-hybridized again with several Wcz and Wcc isolates, a significant signal was obtained for Wcz including LB319 but not for isolates of Wcc (results not shown). Results for four isolates of Wcc hybridized with Wcc tester strain EDHGED are shown in Figure 3.5. The hybridization signal was very clear and distinguishable, such that isolates of Wcc could be distinguished from closely related isolates of Wcz and UWC in the dot blot scheme. Thirty isolates of AG 2-2IIIB also gave significant to strong signals for all but two isolates. UP-PCR products of AG 2-2LP which is closely related to AG 2-2IIIB subgroup gave a

Figure 3.1 UP-PCR banding profile for isolates of *Rhizoctonia solani* AG 1-IB (lanes 1-12) and AG 2-2IIIB (lanes 13-19). L is the mass ladder (1 kb ladder from NEB).

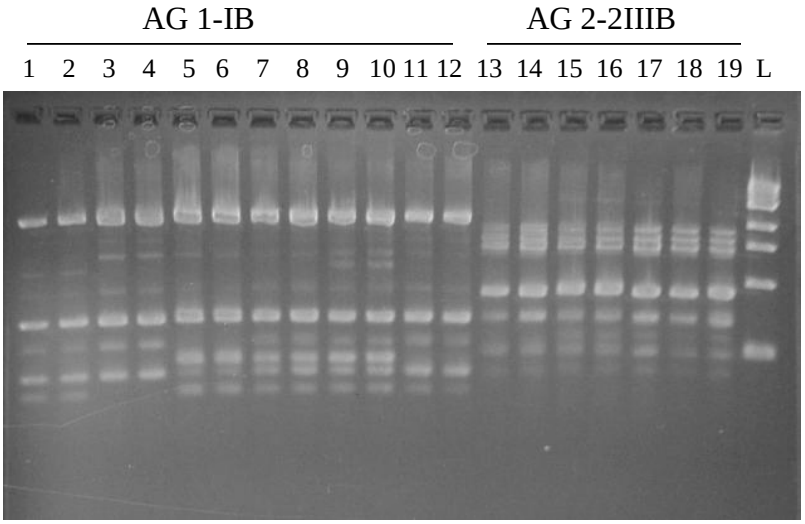
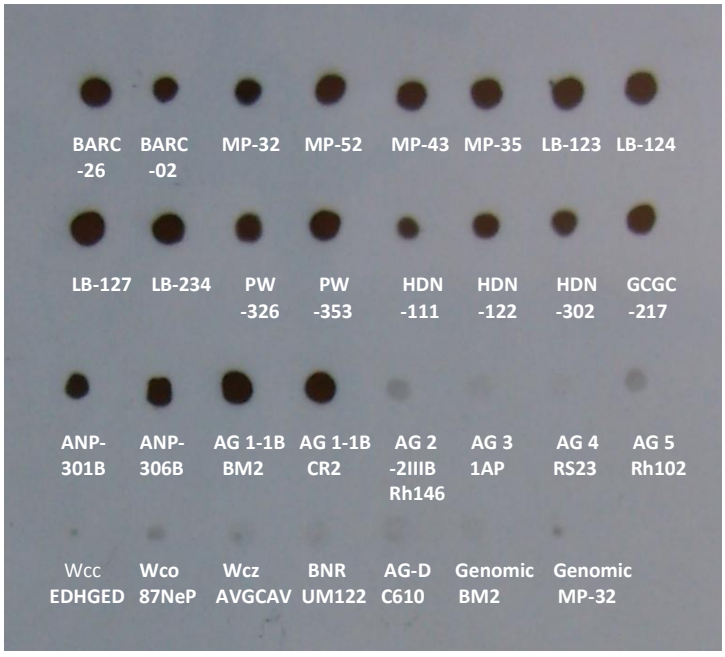


Figure 3.2 Cross blot hybridization of UP-PCR products of AG 1-IB isolates listed in Table 2.1. The labeled probe was total UP-PCR products amplified with primer L21 for AG 1-IB tester strain BM2. The strong signals shown in the photo of radiography are for isolates of AG 1-IB. Isolate names and tester strains on the radiography image. Last two blots are genomic DNA of the probe isolate and MP32.



weak signal similar to other non-homologous AGs in the dot blot scheme. UP-PCR was performed again for the two isolates (ANP309A and LB325) which did not give a strong hybridization signal for AG 2-2IIIB probe. The PCR products were resolved in a 1.7% agarose gel to make sure DNA was amplified and bands were present. Cross-hybridization performed with new UP-PCR products gave a significant signal when hybridized with the AG 2-2IIIB tester strain. Labeled probe of AG 5 made with strain Rh102 produced a significant signal for the AG 5 isolate in the sample (LB204) and a weak or no signal for other AGs.

Figure 3.3 Cross blot hybridization of UP-PCR products of AG 1 subgroups. The labeled probe was prepared using UP-PCR products with UP primer L21 for AG 1-IB strain BM2. Column 1 and 3 represent AG 1-IA and -IC tester strains. Column 2 and 4 are AG 1-IB strains CR1 and BM2. Column 5 has AG 2-2IIIB DNA products. Each column represents three replicates of each strain.

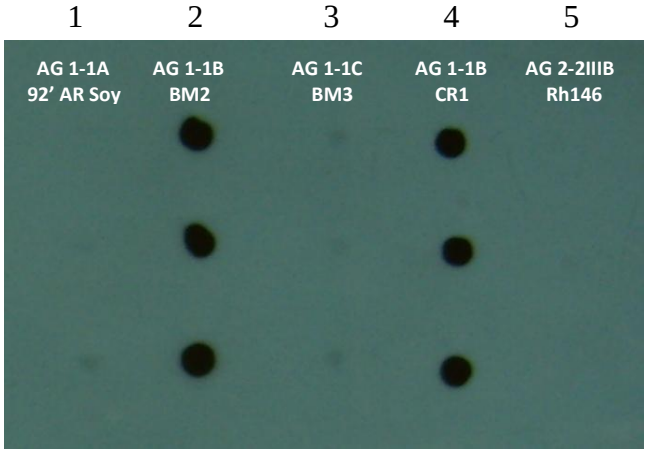


Figure 3.4 Cross blot hybridization of UP-PCR products of *W. circinata* isolates listed in Table 2.1 with a *W. circinata* var. *zeae* (Wcz) tester strain. The labeled probe was prepared for total UP-PCR products with UP primer L21 for Wcz strain AVGCAV. Isolate names and tester strains are indicated in the radiography image.

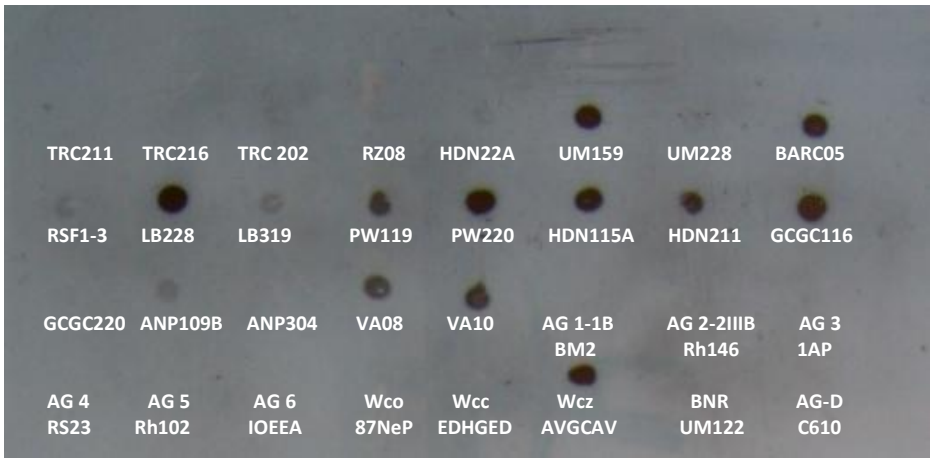


Figure 3.5 Cross blot hybridization of UP-PCR products of *W. circinata* var. *circinata* (Wcc) isolates listed in Table 2.1. The labeled probe was prepared for total UP-PCR products with UP primer L21 for Wcc strain EDHGED. Isolate names and tester strains are indicated on the radiography film. Each strain is replicated three times.

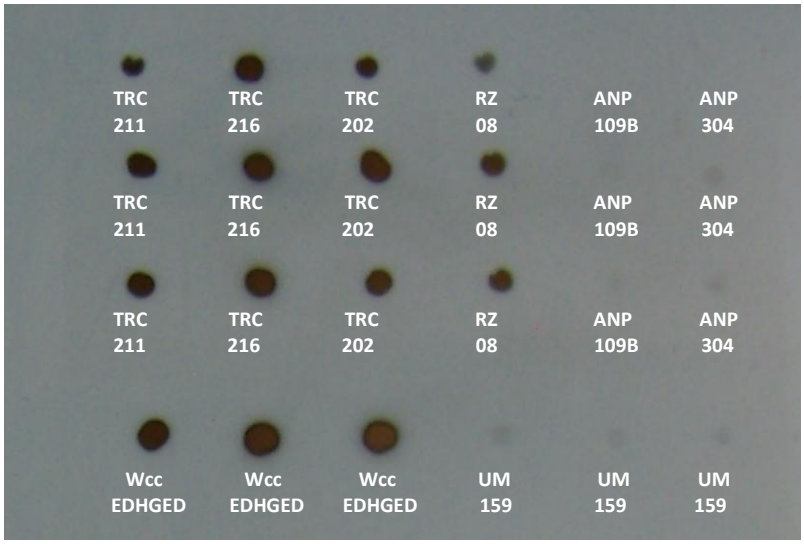
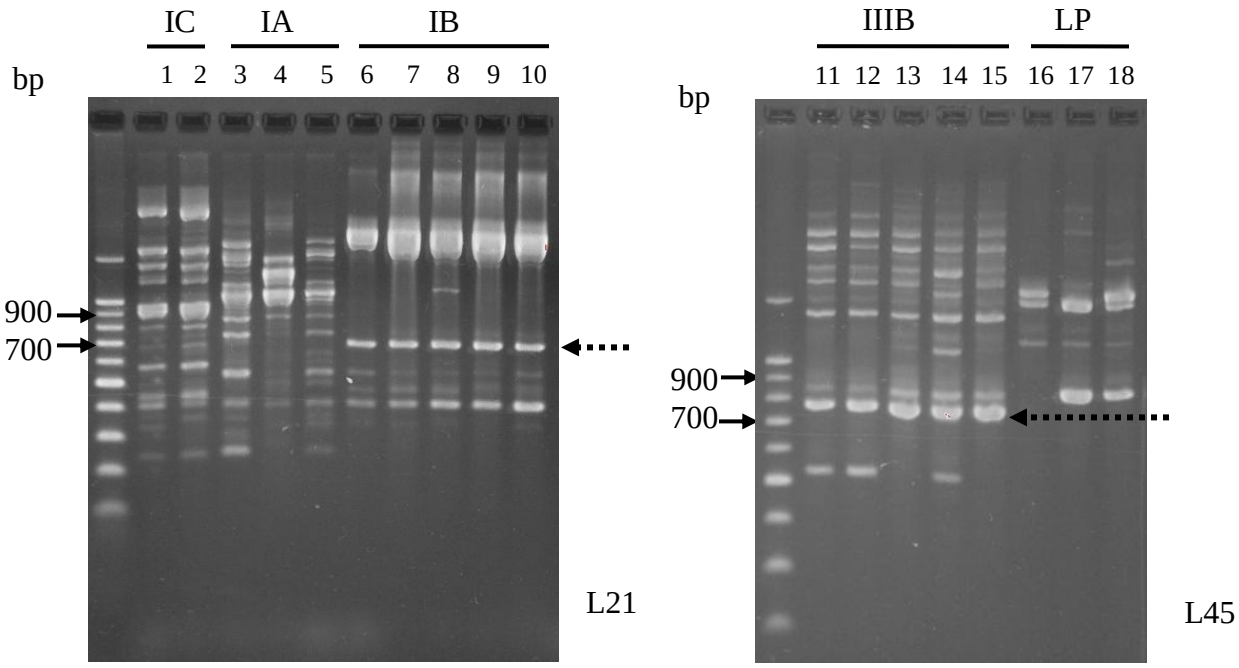


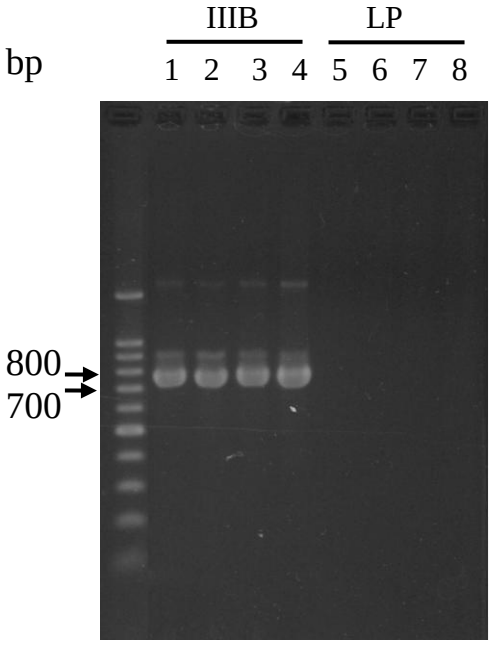
Figure 3.6 UP-PCR banding patterns with primers L21 and L45 for *R. solani* AG 1 and AG 2 isolates respectively. Lanes 1-2, AG 1-IC isolates; Lanes 3-5, AG 1-IA isolates; Lanes 6-10, AG 1-IB isolates; Lanes 11-15, AG 2-2IIIB isolates; Lanes 16-18, AG 2-2LP isolates. The dashed arrow indicates the molecular marker selected for sequencing.



UP-PCR SCAR markers

UP-PCR carried out with DNA of AG 2-2IIIB isolates produced a unique marker for the L45 primer (Figure 3.6) which could distinguish the -2LP isolates from the -2IIIB isolates. UP primer L21 generated a unique marker for AG 1-IB isolates distinguishing AG 1-IA and -IC (Figure 3.6). One of the objectives of this study was to identify unique fragments which were less than 1000 bp in length so as to make sequencing easy. Though other UP primers also generated DNA fingerprinting patterns which were more or less unique for each AG subgroup, UP primers L45 and L21 gave the best unique markers for AG 2-2IIIB and AG 1-IB isolates respectively. The specific primer pairs listed in Table 3.4 were designed from sequencing data

Figure 3.7 PCR products generated using primer pair L45-A/C for *R. solani* AG 2-2 isolates. The annealing temperature used was 63°C. Lanes 1-4, AG 2-2IIIB isolates and lanes 5-8, AG 2-2LP.



of UP-PCR markers shown by dashed arrows in Figure 3.6. The UP-PCR marker with UP primer L45 for AG 2-2IIIB isolates had a length of 734 bp while the marker for AG 1-IB generated by primer L21 was 639 bp in size. The specific primer pair L45-A/C and L21-F/G (Table 3.4) produced fragments only for AG 2-2IIIB (Figure 3.7) and AG 1-IB (Figure 3.9) isolates respectively when amplified by PCR. The different annealing temperatures (58-63°C) gave similar results for both primer pairs by generating bands for either AG 2-2IIIB isolates or AG 1-IB isolates. No bands were produced for closely related AG subgroups of AG 2-2IIIB (2-2LP and 2-1) and AG 1-IB (IA and IC) even at the low annealing temperature of 58°C. The specific primer pairs were used to amplify fungal DNA of other *R. solani* AGs, *R. cerealis* and varieties of *W. circinata* at 63°C annealing temperature but did not yield any amplification products (Figure 3.8 and 3.10).

Figure 3.8 PCR using primer pair L45-A/C for different *Rhizoctonia* species and AGs. Lanes 1-3, AG 2-2IIIB isolates; Lane 4, AG 2-1; Lane 5, BNR isolate UM122; Lane 6, *R. cerealis*; Lanes 7-10, *W. circinata* varieties; Lanes 11-18, *R. solani* AG 8, AG 6, AG 5, AG 4, AG 3, AG 1-IC, AG 1-IB, AG 1-IA.

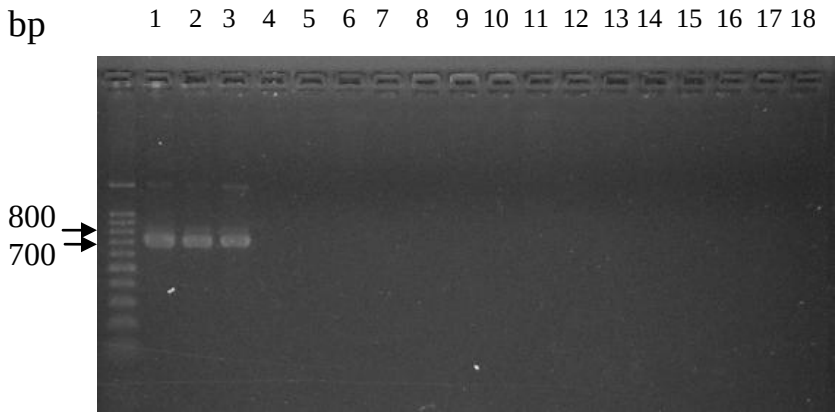


Figure 3.9 PCR products generated by primer pair L21-F/G for *R. solani* AG 1-1A, IB and IC isolates. The annealing temperature of the reaction was 63°C. Lanes 1-5, AG 1-IB isolates; lanes 6-7, AG 1-IC and lanes 8-9, AG 1-IA.

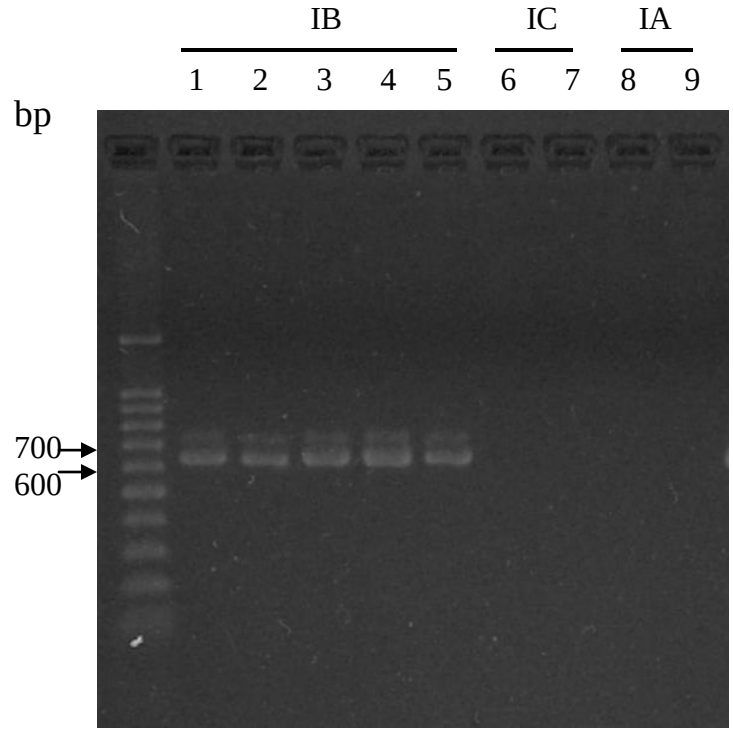
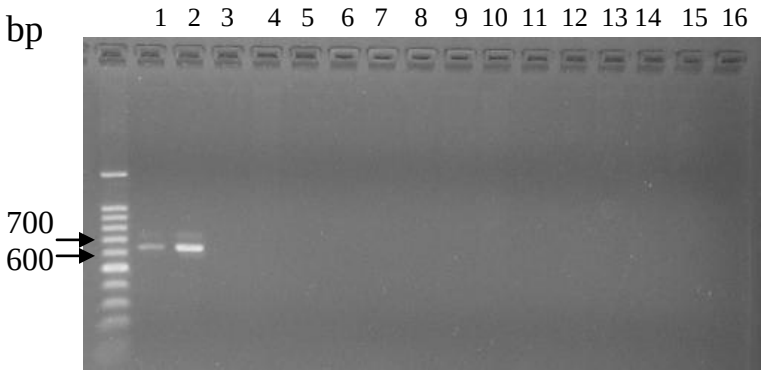


Figure 3.10 PCR using primer pair L21-F/G for different *Rhizoctonia* species and AGs. Lane 1-2, AG 1-IB; Lane3, BNR isolate UM122; Lane 4, *R. cerealis*; Lanes 5-8, *W. circinata* varieties; Lanes 9-16, *R. solani* AG 8, AG 6, AG 5, AG 4, AG 3, AG 2-2IIIB, AG 2-2LP, AG 2-1.



Discussion

UP primer L21 was selected for cross blot hybridization over others since UP-PCR band analysis with L21 could better resolve *Rhizoctonia* isolates into their genetically related groups (Chapter 2). This study shows *R. solani* and *W. circinata* var. *circinata* isolates display a high variability for UP-PCR and hence cross-hybridization of UP-PCR products can be used as an identification tool of unknown *Rhizoctonia* isolates to their AGs and AG subgroups. In particular, UP-PCR hybridization is very useful to rapidly screen a large number of *Rhizoctonia* isolates for classification into their AGs and AG subgroups. The success of this assay heavily depends on the synthesis of efficient probes and high-quality UP-PCR fragments. Similarly the target UP-PCR products also need to consist of a good set of DNA fragments for each isolate to produce a strong signal. One possible reason for not getting a significant hybridization signal for a few isolates the first time the protocol was performed may be due to partial amplification of PCR products. In addition to cross-hybridization, the considerable variability of UP-PCR products among different AGs or AG subgroups enables the use of this method to classify different AGs and AG subgroups by visual examination of PCR fingerprints. Lübeck and Poulsen showed 11 tester isolates of *R. solani* belonging to different AGs and AG subgroups had very different UP-PCR banding profiles with primer L15/AS19 (14). The only exceptions were between AG 2-1 and AG 2-t which shared most of their bands. Visual comparison of aligned UP-PCR products have been used for identification of different *Trichoderma* strains by Lübeck

et al. (16). Species designations for 43 out of 44 unknown *Trichoderma* strains could be assigned by comparing unknown banding profiles with UP-PCR banding patterns obtained from reference strains. The visual comparison is difficult when the PCR profiles for two genetically different strains are too similar or if strains within the same genetically related group has varying banding profiles. These drawbacks of visual comparison can be overcome using the cross-hybridization method (16). Lübeck and Poulsen used cross-hybridization to identify 16 isolates of *R. solani* from diseased sugar beet and potato (14). Cross-hybridization was performed with different tester strains using products of a single primer L15/AS19. Testers of AG subgroups that are homologous with unknown isolates gave strong or significant signals whereas testers of different AGs and AG subgroups gave weak or no signals. These results largely agree with our study which found UP-PCR cross-hybridization can resolve unknown isolates up to their AG subgroup level.

DNA/DNA cross-hybridization between even closely related strains of fungi has been found to be low (12; 14). Since different *Rhizoctonia* AGs and AG subgroups have low sequence complementarity, cross-hybridization can be used for the designation of isolates into these genetic entities (5; 12; 25). According to Kuninaga, DNA hybridization percentages for *Rhizoctonia* isolates falling into different AGs is ≤ 15% while members within an AG mostly have very high (≥ 90%) hybridization percentages (12). Isolates between most subgroups show less than 60% hybridization values except for AG 2-2 subgroups (2IIIB, 2IV and 2LP) which are about 70%. Lübeck and Poulsen compared DNA/DNA hybridization values with UP-PCR cross-hybridization values to estimate the percentage of genomic similarity (expressed as a DNA hybridization value) from the intensity of signal (14). According to them a strong UP-PCR signal seems to indicate a DNA hybridization value of >75%. A significant signal probably represents a 60-75% DNA hybridization value, and a weak signal indicates a DNA hybridization value of approximately 40-60%. No signal for UP-PCR cross blot hybridization is obtained when DNA hybridization is < 40%. The authors used the exposure time of the X-ray films to hybridization membranes in order to derive the signal strength. However, Lübeck and Poulsen do not explain how they derived the relationship between UP-PCR signal strength and DNA hybridization value. It is not possible to compare these results directly with present study since Lübeck and Poulsen used a radioactive detection method for signal development which required exposing autoradiography films from 30 min to overnight. The present study used a highly sensitive

chemiluminescent detection method by exposing X-ray films for 2-10 min. Most isolates gave strong hybridization signals for homologous probes and very weak to no signals for partially homologous or probes lacking homology.

Rhizoctonia species are readily isolated from diseased plants and soils (18). They grow relatively faster in water agar than most fungi and therefore can be easily separated from other fungi to obtain pure cultures. However, disease symptoms, cultural morphology and a microscopic view of different AGs of *Rhizoctonia* can be similar and diagnosis based on these characters is not reliable. Therefore, PCR based amplification of DNA by specific primers is useful to identify AGs and AG subgroups of *Rhizoctonia* isolates rapidly. The specific primer designing was based on identifying a unique band present in all isolates of a target AG type and absent in all other AGs and AG subgroups. Specific primer testing was done with all possible *Rhizoctonia* species and AGs that have been reported to infect turfgrasses, to make sure no PCR product was generated from DNA of non-target organisms. *R. solani* AG 1 through AG 6 have been reported from turfgrasses (4; 22; 26). *W. circinata* (*R. circinata*) varieties *zeae, oryzae* and *circinata* are also responsible for different patch diseases of turf (8; 22). The main binucleate pathogen to cause *Rhizoctonia* blight is *R. cerealis* (4). In addition, there are numerous binucleate *Rhizoctonia*-like species isolated from turfgrasses (3; 4; 10). Several peer reviewed articles indicate the successful identification of *Rhizoctonia* isolates by PCR based techniques (11; 20; 23). Salazar et al. (2000) developed specific primers based on ITS sequence data which could amplify *R. solani* AG 2-1, AG 2-2 and AG 2-3 and ecological group AG 2-t which included isolates from tulip plants. However, these primers were not capable of separating AG 2-2 cultural types AG 2-2IIIB, AG 2-2IV and AG 2-2LP. Toda et al. (2004) designed specific primers from a unique product of RAPD banding patterns to specifically identify AG 2-2LP isolates from diseased turfgrass. They first amplified pure DNA of three AG 2-2 types to confirm the ability of the specific primers to amplify a single DNA fragment for LP isolates only. Then they extracted DNA from leaf sheaths with large patch disease and performed PCR. PCR products were obtained only from leaf sheaths exhibiting large patch and not from healthy leaf sheaths.

Previous studies have revealed *R. solani* AG 1 and AG 2-2 to dominate cool-season turfgrasses (17; 26). The survey conducted in our study on brown patch infected cool-season grasses identified *R. solani* AG 2-2IIIB and AG 1-IB as the most common pathogens in Virginia

and Maryland (Chapter 2). Therefore specific primers were developed to identify these two *R. solani* subgroups. The specific primers L21-F/G and L45-A/C, which were designed to identify AG 1-IB and AG 2-2IIIB groups respectively, did not generate any bands for non-target isolates of other *R. solani* AGs, *R. cerealis*, or *W. circinata* (Figures 3.8 and 3.10). Further, no PCR products were generated for *R. solani* AG 1 types -IA or -IC or AG 2-2LP even at the low annealing temperature of 58°C. These culture types are closely related to the target AG types and therefore indicate the uniqueness of the UP-PCR band that was present in the target isolates from AG 1-IB and AG 2-2IIIB. Further tests are needed using DNA extracted from diseased turfgrass leaves exhibiting brown patch and healthy turfgrass leaves to ascertain whether the specific primers L21-F/G and L45-A/C can distinguish AG 1-IB and AG 2-2IIIB groups from other organisms found on infected and non-infected leaves. PCR based identification of plant pathogens is preferred since primers can amplify even small quantities of target organism's DNA. Toda et al. reported specific primers designed for LP isolates that could amplify and generate a PCR product when the DNA concentration was higher than 1 ng/µl (23). Accordingly, our specific primers may also have the potential to amplify a PCR product from DNA harvested from brown patch diseased leaves.

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CHAPTER 4

In vitro sensitivity of *Rhizoctonia* isolates collected from turfgrasses to selected fungicides

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ABSTRACT

Different *Rhizoctonia* species and anastomosis groups (AGs) have been reported to show variable sensitivity reactions to different commercial fungicide formulations. Forty seven isolates of *Rhizoctonia* collected from turfgrasses were tested *in vitro* for sensitivity to three formulated fungicides, namely, iprodione, triticonazole and pyraclostrobin. *Rhizoctonia* isolates represented 10 isolates each of AG 1-IB, AG 2-2IIIB, binucleate *Rhizoctonia*-like fungi (BNR) and *W. circinata* var. *zeae* (Wcz) from different locations. Isolates tested also represented one isolate of AG 5, four isolates of *W. circinata* var. *circinata* (Wcc) and two undesignated *W. circinata* (UWC) isolates. Potato dextrose agar medium was added with each fungicide to obtain concentrations at 0, 0.1, 1, 10 and 100 mg a.i./l. Effective concentration for 50% inhibition of radial growth (EC_{50}) values were determined for each isolate x fungicide combination. *Waitea circinata* isolates were moderately sensitive to iprodione while isolates of *R. solani* and BNR were extremely sensitive. Isolates of AG 2-2IIIB showed less sensitivity to triticonazole than other *Rhizoctonia* isolates. Isolates of BNR varied in inhibition of growth for triticonazole, exhibiting extreme to moderate sensitivity. Isolates of *W. circinata* var. *zeae* were moderately sensitive to pyraclostrobin while most of the other isolates were extremely sensitive.

Introduction

The form genus *Rhizoctonia* consists of a diverse collection of about 120 species (22). Their teleomorphs belong to different families, orders and even classes. They have a wide host range spanning from crop plants to ornamentals (22; 26). Several of these species are known to infect turfgrass hosts (4; 25). Out of these, the most abundant and most studied species, *R. solani* Kühn (teleomorph: *Thanatephorus cucumeris* (Frank) Donk), is responsible for brown patch on cool-season grasses and large patch on warm-season grasses. Two closely related species, *R. zeae* Voorhees (teleomorph: *Waitea circinata* var. *zeae* Warcup and Talbot) and *R. oryzae* Ryker and Gooch (teleomorph: *W. circinata* var. *oryzae* Warcup and Talbot*)* are responsible for leaf and sheath spot of turfgrasses. They are presumably less prevalent than to *R. solani* since no reports of major disease outbreaks by these two pathogens have been reported. The most recently identified *Rhizoctonia* species, *Waitea circinata* var. *circinata* (*Rhizoctonia circinata*) causes brown ring patch especially on golf putting greens. In 2003, brown ring patch was first reported in eastern Washington, USA on annual bluegrass (9). All the previously mentioned *Rhizoctonia* species are multinucleate. *Rhizoctonia cerealis* Van der Hoeven (teleomorph: *Ceratobasidium cereale* Murray and Burpee), which is responsible for yellow patch, is a cool weather pathogen with binucleate cells. There are numerous reports of other binucleate *Rhizoctonia*-like fungi (BNR) isolated from turfgrass swards and soils (4; 17; 18). These differ from *R. cerealis* in morphological or physiological features as well as anastomosis reactions.

Anastomosis or hyphal fusion reactions among isolates of *Rhizoctonia* have been used traditionally to group *R. solani* and other multinucleate and binucleate *Rhizoctonias* into genetically isolated groups. Isolates which anastomose with each other are somatically and genetically more related to each other than isolates which do not anastomose (5; 21).

Many turfgrasses are susceptible to most of the above mentioned species of *Rhizoctonia* and therefore have the potential to be infected by several species and anastomosis groups (AGs) of *Rhizoctonia* simultaneously. Within *R. solani*, six AGs have been reported to cause brown patch in turfgrass. Out of those AG 1, AG 2-2 and AG 4 are the most commonly reported groups (25; 31). On golf greens and fairways application of fungicides is a common and routine practice to control *Rhizoctonia* diseases. To a lesser extent, athletic fields and home lawns may also employ fungicides in managing *Rhizoctonia* diseases. In a case of multiple infections by different *Rhizoctonia* species and AGs, successful control of *Rhizoctonia* blight requires fungicides with

activity against all species and genetically isolated populations that are present. *In vitro* studies have indicated differential responses by isolates and species to most of the recommended fungicides (4; 12; 13). Since it is unlikely to find a single broad spectrum fungicide to control all *Rhizoctonia* species and isolates, adequate control of this disease complex requires not only accurate identification of the pathogen but also information about the effectiveness of recommended fungicides.

Rhizoctonia solani, *R. zeae* and BNR have different teleomorphs and therefore, represent non-interbreeding populations. *Waitea circinata* var. *circinata* is a new pathogen, identified recently from golf putting greens (9). These species have distinct morphology, physiology, virulence and genetic constitution (13; 30). For instance isolates of BNR have been reported to be weakly pathogenic or non-pathogenic on turfgrasses compared other *Rhizoctonia* species (11; 17). Further, *R. solani* is a species complex with several genetically different subpopulations (8; 15; 24). Therefore, we hypothesized that different *Rhizoctonia* species, AGs and AG subgroups have different levels of fungicide sensitivity. To test this, *in vitro* growth differences in response to several commonly used fungicides were studied for isolates of binucleate and multinucleate *Rhizoctonia* species collected from turfgrasses.

Materials and Methods

Forty seven isolates of *Rhizoctonia* (Table 4.1) collected from turfgrasses were tested *in vitro* for sensitivity to three commonly used fungicides. *Rhizoctonia* isolates represented 10 isolates each of AG 1-IB, AG 2-2IIIB, binucleate *Rhizoctonia*-like fungi (BNR) and *W. circinata* var. *zeae* (Wcz) from different geographies (Table 4.1). Isolates tested also represented one isolate of AG 5, four isolates of *W. circinata* var. *circinata* (Wcc) and two unknown *W. circinata* (UWC) isolates. The formulated fungicides, triticonazole (Trinity), iprodione (Iprodione Pro 2SE), and pyraclostrobin (Insignia) all manufactured by BASF Corporation (Florham Park, New Jersey, USA) were used in this study. The experiment was conducted in 9 cm petri dishes containing 15 ml of PDA medium amended with the relevant fungicide. The autoclaved PDA medium, after cooling to 50°C, was amended with a fungicide to obtain the following concentrations: 0.1, 1, 10 and 100 mg a.i./l. Control petri dishes were not amended with fungicide. Fungicide amended and control petri dishes were inoculated with 6 mm mycelial plugs cut from the margin of actively growing PDA cultures of the isolates of *Rhizoctonia*.

Table 4.1 Geographic origin, host and anastomosis group of isolates used in this study

Isolate	Host	Origin	Species	AG/subgroup
UMTRC 114	Tall fescue	Beltsville, MD	Rs	AG 2-2IIIB
RS 6	CBG/ABG	Blacksburg, VA	Rs	AG 2-2IIIB
GC 32C	CBG/ABG	Blacksburg, VA	Rs	AG 2-2IIIB
GC 20C	CBG/ABG	Blacksburg, VA	Rs	AG 2-2IIIB
BSF 42	Tall fescue	Richmond, VA	Rs	AG 2-2IIIB
BSF 90	Tall fescue	Richmond, VA	Rs	AG 2-2IIIB
LB 312	Tall fescue	Leesburg, VA	Rs	AG 2-2IIIB
HDN 208By	Tall fescue	Herndon, VA	Rs	AG 2-2IIIB
GCGC 303	Tall fescue	Leesburg, VA	Rs	AG 2-2IIIB
ANP 202B	Tall fescue	Annapolis, MD	Rs	AG 2-2IIIB
BARC 26	Tall fescue	Beltsville, MD	Rs	AG 1-IB
BARC 02	Tall fescue	Beltsville, MD	Rs	AG 1-IB
MP 32	Tall fescue	Blacksburg, VA	Rs	AG 1-IB
MP 43	Tall fescue	Blacksburg, VA	Rs	AG 1-IB
LB 123	Tall fescue	Leesburg, VA	Rs	AG 1-IB
LB 234	Tall fescue	Leesburg, VA	Rs	AG 1-IB
PW 326	Tall fescue	Woodbridge, VA	Rs	AG 1-IB
HDN 111A	Tall fescue	Herndon, VA	Rs	AG 1-IB
GCGC 217	Tall fescue	Leesburg, VA	Rs	AG 1-IB
ANP 301B	Tall fescue	Annapolis, MD	Rs	AG 1-IB
LB 204	Tall fescue	Leesburg, VA	Rs	AG 5
UMTRC 122	Tall fescue	Beltsville, MD	BNR	unknown
BARC 17	Tall fescue	Beltsville, MD	BNR	unknown
SF 224	Tall fescue	Richmond, VA	BNR	unknown
LB 226	Tall fescue	Leesburg, VA	BNR	unknown
PW 154	Tall fescue	Woodbridge, VA	BNR	unknown
PW 341	Tall fescue	Woodbridge, VA	BNR	unknown
HDN 209	Tall fescue	Herndon, VA	BNR	unknown
HDN 325B	CBG	Herndon, VA	BNR	unknown
GCGC 202A	Tall fescue	Leesburg, VA	BNR	unknown
ANP 107	Tall fescue	Annapolis, MD	BNR	unknown
BARC 05	Tall fescue	Beltsville, MD	Wcz	WAG-Z
LB 319	Tall fescue	Leesburg, VA	Wcz	WAG-Z
PW 220	Tall fescue	Woodbridge, VA	Wcz	WAG-Z
PW 119	Tall fescue	Woodbridge, VA	Wcz	WAG-Z
HDN 115A	Tall fescue	Herndon, VA	Wcz	WAG-Z
GCGC 116	Tall fescue	Leesburg, VA	Wcz	WAG-Z
VABCH 08	Tall fescue	Virginia Beach, VA	Wcz	WAG-Z
VABCH 10	Tall fescue	Virginia Beach, VA	Wcz	WAG-Z
UMTRC 159	Tall fescue	Beltsville, MD	Wcz	WAG-Z

Table 4.1 Continued

Isolate	Host	Origin	Species	AG/subgroup
LB 228	Tall fescue	Leesburg, VA	Wcz	WAG-Z
GCGC 220	CBG	Leesburg, VA	UWC	WAG
HDN 222A	Tall fescue	Herndon, VA	UWC	WAG
TRC 211	CBG/ABG	Blacksburg, VA	Wcc	WAG
TRC 216	CBG/ABG	Blacksburg, VA	Wcc	WAG
TRC 202	CBG/ABG	Blacksburg, VA	Wcc	WAG
RZ 08	CBG/ABG	Blacksburg, VA	Wcc	WAG

ABG = annual bluegrass; CBG = creeping bentgrass
Rs = *R. solani*; BNR = binucleate *Rhizoctonia*-like fungi; Wcz = W. *circinata* var. *zeae;* Wcc = *W. circinata* var. *circinata*; UWC = unidentified *W. circinata* species

Mycelial plugs were inverted centrally on each petri dish. Four replicate petri dishes per isolate for each fungicide concentration and the untreated controls were incubated in the dark at 27°C. Colonies of fungicide amended dishes were measured along two right angle diameters just before the mycelial mat of the untreated control reached the edge of the petri dish. The diameter of the mycelial plug was subtracted and the average growth of each isolate was determined using the two growth measurements. The percent growth inhibition for each isolate x fungicide combination was calculated using the following formula.

$$\% \text{ Inhibition} = \frac{(\text{diameter of control} - \text{diameter of treated}) \times 100}{\text{diameter of control}}$$

The experiment was repeated once with similar results for most of the isolates. The data reported herein are from the second experiment. Percent growth inhibitions for different concentrations from each isolate were used to derive the effective concentration that caused 50% growth inhibition (EC_{50}) by log-probit analysis using SAS ver. 9.2 (SAS Institute, Cary, NC). The sensitivity scale based on EC_{50} values adopted by Martin et al. and described below was used to compare the sensitivity of the isolates to various fungicides (18). Isolates were considered extremely sensitive if the EC_{50} of a fungicide was less than 1 mg a.i./l, moderately sensitive if the EC_{50} was 1-10 mg a.i./l, and tolerant if EC_{50} exceeded 50 mg a.i /l.

Results

The EC_{50} values obtained by exposing each group of isolates of *Rhizoctonia* to iprodione, triticonazole, and pyraclostrobin are summarized in Table 4.2. All isolates of *R. solani* (AG 1-IB, AG 2-2IIIB and AG 5) and binucleate *Rhizoctonia*-like fungi (BNR) except for GCGC202A, HDN209 and LB226 were extremely sensitive to iprodione (Figure 4.1). The average EC_{50} values for these isolates were < 1 mg a.i./l. The three BNR isolates mentioned above showed EC_{50} values slightly above 1 mg a.i./l and would be considered moderately sensitive. All *W. circinata* isolates were moderately sensitive to iprodione. The EC_{50} values ranged from 1.5 to 2.66 mg a.i/l for these isolates (Table 4.2).

Isolates of AG 1-IB, and *W. circinata* showed extreme sensitivity to triticonazole. EC_{50} values for these isolates were ≤ 0.25 mg a.i./l (Table 4.2). The single isolate of AG 5 had EC_{50} value of 0.4 mg a.i./l. Isolates of AG 2-2IIIB appeared to be moderately sensitive to triticonazole with a mean EC_{50} value of 1.86 mg a.i./l (Table 4.2). BNR isolates showed extreme to moderate sensitivity to triticonazole since EC_{50} values varied from 0.01-3.57 mg a.i./l (Figure 4.2). Forty percent of the BNR isolates tested had EC_{50} values > 1 mg a.i. /l.

Table 4.2 EC_{50} values (mg a.i./l) for isolates of *R. solani* AG 1-IB, AG 2-2IIIB, *W. circinata* and binucleate *Rhizoctonia* like fungi exposed to iprodione, triticonazole and pyraclostrobin

Group	No. of isolates	Iprodione		Triticonazole		Pyraclostrobin	
		Mean	Range	Mean	Range	Mean	Range
R. solani							
AG 1-IB	10	0.46	0.30-0.71	0.09	0.01-0.25	0.17	0.02-0.46
AG 2-2IIIB	10	0.50	0.37-0.70	1.86	0.39-4.18	0.17	0.02-0.42
AG 5	1	0.40	n/a	0.46	n/a	0.59	n/a
W. circinata							
Wcz	10	1.92	1.50-2.51	0.06	0.01-0.14	3.18	0.18-5.34
Wcc	4	2.38	2.01-2.66	0.03	0.02-0.05	1.64	0.36-4.87
UWC	2	2.02	1.62-2.41	0.004	0.002-0.006	0.26	0.14-0.39
BNR	10	0.83	0.38-1.18	1.05	0.01-3.57	0.44	0.05-2.43

Wcz: *W. circinata* var. *zeae*, Wcc: *W. circinata* var. *circinata*, UWC: Unidentified *W. circinata*, BNR: binucleate *Rhizoctonia*-like fungi

Figure 4.1 Sensitivity of isolates of *Rhizoctonia* to iprodione. Bars represent standard error.

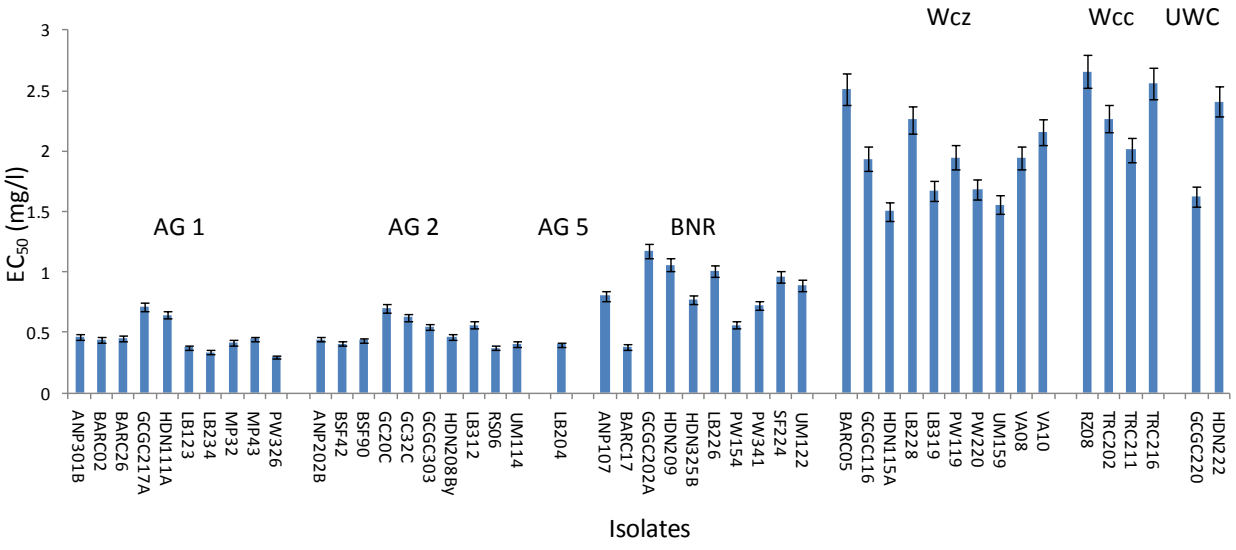


Figure 4.2 Sensitivity of isolates of *Rhizoctonia* to triticonazole. Bars represent standard error.

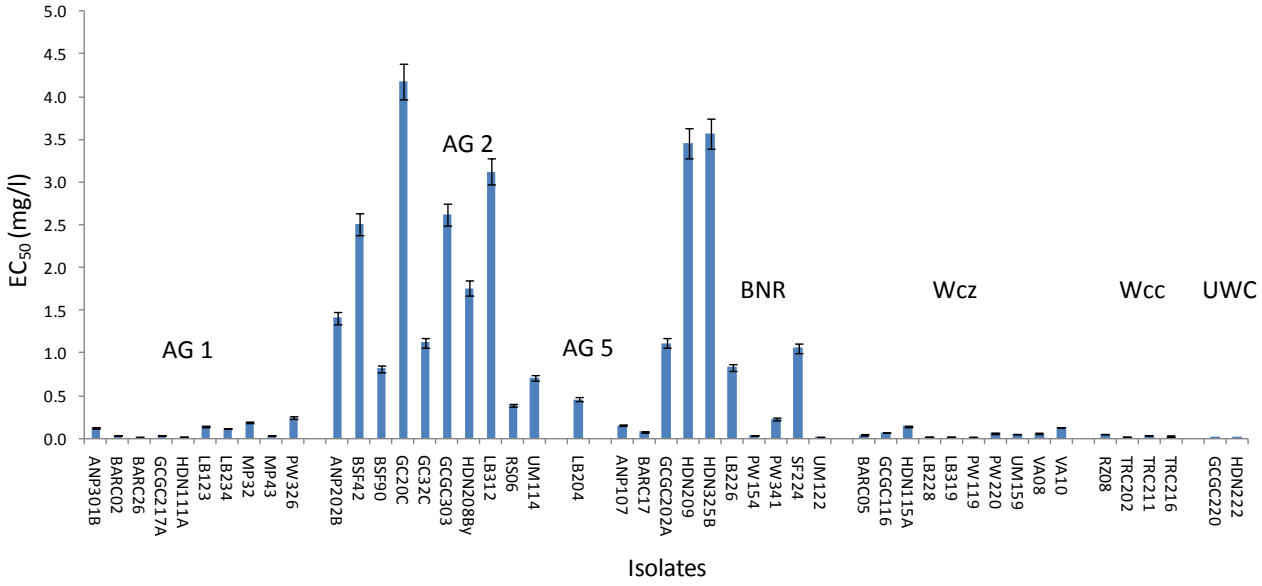
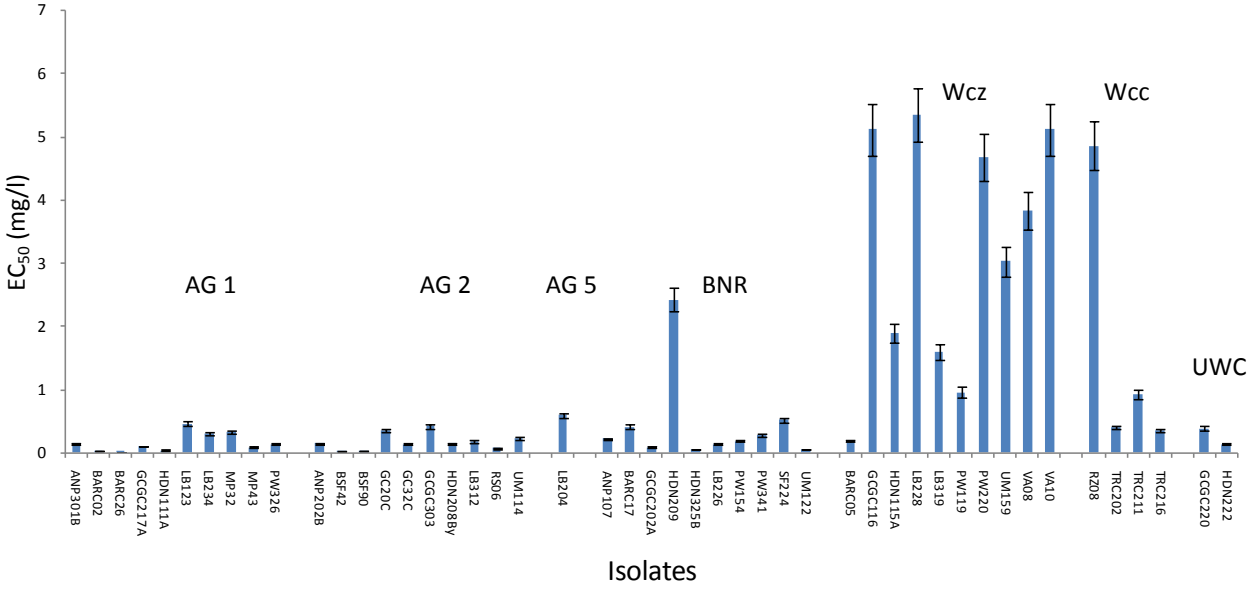


Figure 4.3 Sensitivity of isolates of *Rhizoctonia* to pyraclostrobin. Bars represent standard error.



The reactions of isolates to pyraclostrobin are shown in Figure 4.3 and Table 4.2. Isolates of *W. circinata* var. *zeae* and var. *circinata* were moderately sensitive to pyraclostrobin with average EC_{50} values of 3.18 and 1.64 mg a.i./l respectively. All other groups were extremely sensitive (EC50 < 1 mg a.i./l). However BNR isolate HDN209 had a high EC_{50} of 2.43 mg a.i./l.

Discussion

Iprodione, triticonazole and pyraclostrobin, each belong to different fungicide groups having entirely different modes of actions. Iprodione belongs to the dicarboximide fungicide group and was introduced to the market almost three decades ago to control a wide variety of fungal diseases including brown patch of turfgrasses (23). Previous studies have shown *R. solani* and binucleate *Rhizoctonia* are more sensitive to iprodione than are *R. zeae* and *R. oryzae* (6; 13; 18; 19). However, these studies did not include *W. circinata* var. *circinata*. The results of our study which evaluated isolates of three AGs of *R. solani*, unknown binucleate *Rhizoctonia*-like fungi (BNR), two varieties of *W. circinata* and two unknown *W. circinata* isolates support the previous research showing *W. circinata* isolates are less sensitive to iprodione. According to the sensitivity scale described by Martin et al. (18), our study showed all *R. solani* (*T. cucumeris*)

isolates were extremely sensitive to iprodione while all varieties of *W. circinata* were moderately sensitive. The sensitivity of isolates of BNR to iprodione was in between that of *R. solani* and *W circinata* (Table 4.2). The isolates of AG 1-IB, AG 2-2IIIB, BNR and Wcz represent several different geographic locations (Table 4.1). It seems apparent that geography does not have much influence on the EC_{50} values of the isolates since there were no large deviations of sensitivity among geographically different isolates. However, it would be beneficial to study differences in sensitivity of *Rhizoctonia* isolates from differently managed turf. Golf putting greens are extensively managed with routine fungicide applications while lawns and athletic fields are occasionally or very rarely exposed to fungicides. Our experiment does not include enough isolates from golf putting greens for such a comparison.

The second fungicide triticonazole is a systemic fungicide with a triazole moiety having acropetal movement. Triticonazole has a DMI (demethylation inhibitor) mode of action, meaning that the fungicide disrupts sterol biosynthesis in fungal cell membranes leading to alterations of the structure and disturbances in the division and development of cells. Triticonazole was introduced by the Rhone-Poulenc Ag company in the mid 1990s (3). Studies on other DMI fungicides such as propiconazole, fenarimol, and triadimefon have shown variable results *in vitro* and in field studies for different species and AGs of *Rhizoctonia* (12; 20; 29). There are no previous reports of challenging *Rhizoctonia* isolates with triticonazole *in vitro*. Our study indicated AG 1-IB, AG 5 and all varieties of *W. circinata* were extremely sensitive to triticonazole (EC50 < 1 mg a.i / l). The mean EC_{50} values of isolates of AG 2-2IIIB and BNR indicated moderate sensitivity to triticonazole. It seems the variability of EC_{50} values are similarly not associated with geography. AG 2-2IIIB isolates BSF42 and BSF90 from Richmond, VA showed moderate sensitivity and extreme sensitivity, respectively for triticonazole. EC_{50} values for BSF42 and BSF90 were 2.5 mg a.i./l and 0.82 mg a.i / l respectively. In the same way GC32C showed a high EC_{50} value of 4.18 mg a.i./l while GC20C had a comparatively low EC_{50} value of 1.12 mg a.i./l for triticonazole. Both these isolates were collected from the Virginia Tech turfgrass research center. Kataria et al. tested a single isolate of AG 1, Wco and Wcz against different DMI fungicides *in vitro* (13). Their results showed all three isolates were extremely sensitive to cyproconazole. However, propiconazole, another DMI fungicide, was less effective for the AG 1 isolate (EC_{90} = 47 mg a.i./l) while both Wcz and Wco isolates showed extreme sensitivity ($EC_{90} \leq 2$ mg a.i./l). These results indicate that different DMI fungicides can

have different efficacies against different *Rhizoctonia* (*Thanatephorus* and *Waitea*) genera and AGs.

Pyraclostrobin is a relatively new fungicide for turfgrass disease control. It was first marketed in 2002 by BASF chemical company (2). This fungicide is recommended for control of brown patch, large patch and leaf and sheath spot diseases of turfgrasses. Pyraclostrobin belongs to the strobilurin fungicide group, also referred to as quinone outside inhibitors (QoI). The fungicide inhibits mitochondrial respiration within fungal cells, blocks electron transport at cytochrome bc-1 complex which results in fungal cells that are starved of energy molecule ATP required for growth and disease development (2; 10). Pyraclostrobin has a translaminar movement in plants and no records of resistance have been reported from *R. solani* or *Waitea* species for this fungicide. The present study showed pyraclostrobin was very effective against all *Rhizoctonia* groups other than isolates of Wcz, which were moderately sensitive. However, one isolate of BNR and Wcc also showed moderate sensitivity to the fungicide. Meyer et al. studied *in vitro* sensitivity of an isolate of *R. solani* AG 1-IA from soybean to pyraclostrobin (20). They reported that the AG 1-IA isolate was extremely sensitive to pyraclostrobin with EC_{50} value of 0.094 mg a.i./l. The EC_{50} values of our isolates of *R. solani* were in the range of 0.02-0.59 mg a.i./l. Though we studied closely related AG 1-IB isolates and Meyer et al. used a single AG 1-1A isolate to calculate their EC_{50} for pyraclostrobin, their result falls within the EC_{50} value range we obtained for *R. solani* AG 1 isolates. Several QoI fungicides have shown reduced effectiveness *in vitro* for some pathogens due to site specific mutations (7; 14; 16). Ma et al. reported resistance to azoxystrobin by *Alternaria* species isolated from pistachio in California (16). Azoxystrobin and trifloxystrobin resistant isolates of *Pyricularia grisea* have been identified from perennial ryegrass turf in Illinois (14). *P. grisea* is responsible for gray leaf spot disease in golf courses. Authors of that article, Kim et al. (14) found the resistance was due to a small number of mutations in the fungicide target site, which was the cytochrome b gene.

At the time Martin et al. (18) introduced their fungicide sensitivity classification, the strobilurin fungicide group was not available in the market. Different classifications have been published for this fungicide group. Ma et al., in their study of *Alternaria* species against azoxystrobin, as mentioned in the previous paragraph, designated isolates with EC_{50} values less than 0.01 mg/l as extremely sensitive while isolates with EC_{50} values greater than 100 mg/l were considered tolerant (16). Avenot et al. grouped isolates of *A. alternata* causing late blight of

pistachio into four classes depending on their sensitivity to pyraclostrobin (1). Isolates classified as sensitive had EC_{50} values ranging between 0 and 1 mg/l, weakly resistant isolates had EC_{50} values between 1 and 15 mg/l, intermediate-resistant isolates had EC_{50} values between 15 and 100 mg/l, and highly resistant isolates had EC_{50} values above 100 mg/l. The classification given by Martin et al. (18) more or less agrees with these two fungicide sensitivity scales.

The main aim of this study was to test the hypothesis that different *Rhizoctonia* groups have different levels of fungicide sensitivity. Results of the experiment supported that different *Rhizoctonia* species, AGs and subgroups may respond differently to fungicides *in vitro*. However, *in vitro* results may not correlate well with fungicide control in a greenhouse or in the field. In fact, certain diseases have been reported as not being controlled, or disease incidence increases when fungicides are applied in greenhouse or field trials (19; 27; 28). According to Van der Hoeven and Bollen, an increase of sharp eye spot of rye caused by *R. cerealis* was observed after benomyl treatments in the field even though the pathogen was sensitive to benomyl *in vitro* (28). One explanation may be a decrease in antagonistic microflora present in the environment. Another possibility for *in vitro* and *in vivo* differences may be due to fungicide metabolism by the plant that do not occur in the petri dish. Prevailing climatic conditions also greatly influence the effectiveness of a fungicide by favoring either the plant or the pathogen. Therefore additional greenhouse and field studies with isolates of *Rhizoctonia* species are necessary to confirm the practical value of results obtained by *in vitro* fungicide sensitivity tests.

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CHAPTER 5

Conclusions and Future Directions

Several *Rhizoctonia* genera (*Thanatephorus*, *Ceratobasidium* and *Waitea*) and anastomosis groups (AGs) within *R. solani* (*T. cucumeris*) are responsible for *Rhizoctonia* blight on turfgrasses. The survey carried out in different locations of Virginia and Maryland revealed *R. solani* AG 2-2IIIB to dominate cool-season turfgrasses and cause brown patch. Thirty five percent [35% (30/86)] of the analyzed sample consisted of isolates of AG 2-2IIIB. This was followed by isolates of AG 1-IB [21% (18/86)] and binucleate *Rhizoctonia*-like fungi (BNR) [19% (16/86). *Waitea circinata* isolates represented 24% (21/86) of the sample. Out of the *Waitea* species, *W. circinata* var. *zeae* was the dominant pathogen.

We tested the ability of three molecular methods to group and identify isolates of genetically diverse *Rhizoctonia* from turfgrass. One method, ribosomal DNA internal transcribed spacer (rDNA-ITS) region analysis, analyzed the sequence of a single site while other two methods, universally primed polymerase chain reaction (UP-PCR) and amplified fragment length polymorphism (AFLP), analyzed the DNA fragment polymorphism of multiple sites. All three techniques could identify unknown isolates to their AG or AG subgroup level, and clades formed, largely corresponded to the AG or AG subgroup of the isolates tested.

Future research should apply these techniques to investigate the genetic structure of *Rhizoctonia* pathogens on extensively managed golf putting greens verses lawns and athletic fields, which are occasionally sprayed with fungicides. It is important to have an understanding of the fungicide history and cultural practices at each site for making inferences of the molecular analysis with regards to the genetic structure of pathogens. One limitation of our study was the unavailability of this information. Another research area would be the host-specificity of different *Rhizoctonia* species, AGs and AG subgroups. We sampled three golf course putting greens having creeping bentgrass and annual bluegrass in Blacksburg, Leesburg and Herndon. All isolates were designated *R. solani* AG 2-2IIIB. However the number of sampled isolates were limited to seven for all three sites which is not sufficient to make any inferences on the host-specificity of AG 2-2IIIB. Similarly, all AG 1-IB isolates came from tall fescue grass. Future work should concentrate on sampling more isolates from different turfgrass species to determine host specificity. Martin and Lucas assigned most of the isolates collected from foliage

of tall fescue in Raleigh, North Carolina, to AG 1 (8). A study done in Maryland identified 70% (21/30) of the isolates causing brown patch on perennial ryegrass belonged to *R. solani* AG 1-1A whereas all the isolates from colonial bent were AG 2-2IIIB (12). Two previous studies in Texas (6) and South Carolina (5) assigned *Rhizoctonia* isolates from diseased leaf sheaths of St. Augustinegrass to AG 2-2. These preliminary data indicate there is a possibility of host-specificity of *Rhizoctonia* isolates. However large samples from many locations needed to be investigated.

Both rDNA-ITS and UP-PCR analyses revealed six isolates of *W. circinata* (UWC) which did not group with already known *W. circinata* var. *zeae, W. circinata* var. *oryzae* or *W. circinata* var. *circinata*. The ITS sequences of these isolates were closely related to *W. circinata* var. *agrostis* (11) from Japan and *W. circinata* var. *prodigus* (7) from Florida. This indicates the possibility of the existence of low level populations of unidentified varieties of *W. circinata* causing disease on turfgrasses. Koch's postulates need to be performed on the isolates of UWC to characterize these further. It may be useful to survey golf courses from northern Virginia to ascertain how abundant and widespread the unassigned *W. circinata* isolates are since one isolate, namely GCGC 220, came from a putting green from this area.

Zhang and Dernoeden (12) reported that genetic structure of *Rhizoctonia* pathogens did not change over seasons, in a study carried out in Maryland. However they sampled only one site, and used time consuming conventional techniques to characterize *Rhizoctonia* species. It is important to study many sites, especially on golf courses to understand whether *Rhizoctonia* populations change over seasons. The molecular techniques identified in our study can be used to characterize a large number of unknown isolates of *Rhizoctonia* rapidly. Another important aspect is to understand the population dynamics of *Rhizoctonia* species and AGs at different time periods within a season. For instance, the cool weather pathogen *R. cerealis* infects turfgrass during late spring to early summer, whereas *R. solani* prefers warm humid mid-summer conditions. Knowledge of *Rhizoctonia* population dynamics in space and time is important for disease management.

BNR isolates most probably represent more than one AG or AG subgroup since cladistic analysis placed a few isolates separate from the rest of the BNR clade. The AFLP method that was used seems to be less suitable for clustering isolates of *Rhizoctonia* representing different genera and is better suited to studying closely related groups within a species. It may be possible

to obtain reliable results for genetically diverse set of isolates by screening many AFLP primers and selecting the suitable ones for the detailed analysis (10). The molecular analysis of *Rhizoctonia* isolates did not reveal any correlation of geographic origin with clusters that were formed based on the molecular techniques used.

Cross-hybridization of UP-PCR products generated by a single primer could identify most *Thanatephorus cucumeris* (*R. solani*) and *W. circinata* isolates into their anastomosis subgroup level. Cross blot hybridization is a useful molecular technique to derive AGs of many unknown *Rhizoctonia* isolates within a short period of time. The success of cross blot hybridization depends largely on the efficiency of the labeled probe and therefore labeled tester strains should be tested for efficacy prior to hybridizing with target organisms. Grouping unknown *Rhizoctonia* isolates by traditional anastomosis reactions has certain limitations such as difficulty in interpreting results and the extended time needed for observation of the reactions. Further, anastomosis cannot be used to identify AG subgroups since isolates within an AG most of the time anastomose each other. UP-PCR cross blot hybridization can be used as a tool to overcome these problems with anastomosis reactions. Sequenced-characterized amplified regions (SCAR) markers that were developed for AG 1-IB and AG 2-2IIIB could distinguish isolates of these two AG subgroups from other *Rhizoctonia* species and AGs. The PCR amplification of pure DNA resulted in a DNA fragment constant with AG 1-IB or AG 2-2IIIB but not other AGs. It is useful to test the ability of these specific primer pairs (AG 1 F/G and AG 2 A/C) to identify isolates of relevant AG subgroups from DNA of diseased turfgrass leaves. *R. solani* AG 1-IB and AG 2-2IIIB are important pathogens causing brown patch on turfgrasses. The isolates of these two AG subgroups represented >55% (48/86) of the sample.

Another molecular technique for rapid identification of *Rhizoctonia* species is the analysis of the ITS region. The ITS sequences of unknown isolates of *Rhizoctonia* can be compared with already characterized sequences deposited in publicly accessible databases such as GenBank and DDBJ (DNA database of Japan). Although it is rare to find 100% similar sequences to unknowns via BLAST queries, close matches usually help in correct identification. This study used reference sequences of different AGs from GenBank and analyzed unknown field samples to confirm their identity. However, there are some disadvantages of using public databases. They are primary databases and as a result anyone can submit sequences and sequence accuracy is not checked. Some deposited sequences may have very little information for

meaningful interpretation, and as such reference sequences should be carefully selected for any analysis. Once holotype strains are characterized and ITS sequences are generated, the accuracy of banked sequences can be checked by constructing trees with holotype sequences.

Fungicide sensitivity experiment was conducted with 10 isolates each of *R. solani* AG 2-2IIIB, AG 1-IB, *W. circinata* var. *zeae* (Wcz) and BNR. Isolates tested also represented one isolate of AG 5, four isolates of *W. circinata* var. *circinata* (Wcc) and two unknown *W. circinata* (UWC) isolates. Following the classification given by Martin et al (9), the *in vitro* fungicide sensitivity reactions indicated all *W. circinata* varieties [i.e. var. *zeae*, var. *circinata* and UWC] were moderately sensitive to iprodione (EC_{50} between 1-10 mg a.i./l) while isolates of *R. solani* and BNR were extremely sensitive (EC_{50} < 1 mg a.i./l). The DMI fungicide triticonazole exhibited very high control of isolates of *W. circinata*, *R. solani* AG 1-IB and AG 5 (EC_{50} < 1 mg a.i./l). Most of AG 2-2IIIB isolates showed moderate sensitivity (EC_{50} between 1-10 mg a.i./l) while BNR isolates had variable results of extreme sensitivity to moderate sensitivity (EC_{50} between 0.01-3.57 mg a.i./l). Pyraclostrobin showed a high level of control *in vitro* for most of the isolates tested except for Wcz. Most of the Wcz isolates were moderately sensitive to pyraclostrobin (average EC_{50}= 3.18 mg a.i./l). Fungicide sensitivity experiments indicated different AG subgroups of *R. solani* and varieties of *W. circinata* exhibited different sensitivity reactions to tested fungicides. However, sensitivity from a few isolates within an AG subgroup or a *W. circinata* variety deviated largely from the mean from a group. This result may help explain why various fungicides can produce variable control in the field. Further tests are needed to perform with these three fungicides in greenhouse and field trials against *R. solani* and *W. circinata* species to evaluate the impact outside forces have on fungicide efficacy.

It is important to accurately identify and classify *Rhizoctonia* isolates into genetically related groups since some subgroups of *Rhizoctonia* share pathogenic characteristics such as host range, virulence or sensitivity to fungicides (1; 2; 6; 9). Though several *Rhizoctonia* species and AGs can be associated with turfgrasses, usually there is a primary pathogen. This information is very useful for plant breeders who are aiming to develop resistant varieties. Our survey identified Wcc isolates from Virginia Tech turfgrass research center. As previously described, diseased leaf samples from many sites of several adjacent golf courses needed to be characterized to ascertain the abundance and spatial distribution of this species. This information would be useful for turfgrass breeders to screen turf varieties resistant to isolates of Wcc.

Accuracy in distinguishing pathogens at the initiation of disease is important to implement successful disease control measures. The SCAR markers and UP-PCR cross-hybridization techniques are two such molecular tools which have the potential for rapid identification of unknown isolates of *Rhizoctonia*. Our results indicated that *R. solani* AG 2-2IIIB was the established and perhaps dominant anastomosis subgroup causing brown patch in cool-season turfgrasses. The anastomosis reactions on a petri dish or slide cannot be used to differentiate AG subgroups, but the molecular techniques such as sequencing rDNA-ITS regions or UP-PCR fingerprinting profiles used in this study were useful in differentiating isolates of *R. solani* and *W. circinata* to their respective subgroup. Our molecular analysis of *Rhizoctonia* isolates associated with turfgrass diseases provides initial information on the occurrence of a relatively diverse group of *Rhizoctonia* species. This study identified not only widely reported *Rhizoctonia solani* and *Waitea circinata* var. *zeae*, but also lesser known varieties of *W. circinata* and binucleate *Rhizoctonia*-like fungi from the diseased turfgrasses.

The ITS region analysis and fingerprinting techniques employed in this study can be used to understand the genetic structure of *Rhizoctonia* pathogens of turfgrasses. An understanding of genetic structure is necessary in order to compare population structures of *Rhizoctonia* (3). This information is important in epidemiological studies of the *Rhizoctonia* pathosystem (4). Repeated recovery of similar genotypes from the same location over seasons is an indication of limited genetic diversity and asexual breeding of the *Rhizoctonia* species and AGs. If molecular analyses result in new clades from isolates, sampled over time, it is possible that either introduction of new genotypes from outside or sexual reproduction of the existing populations. The sample size of most of the AG subgroups analyzed in our study is not sufficient to make any inferences concerning the contribution of observed genotype diversity to population structure. Though there were no clonal genotypes, it was apparent that most of isolates within an AG subgroup were closely related.

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